Identification of a putative *ampG* ampicillin resistance gene in *Stenotrophomonas maltophilia* OR02

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Abstract

Stenotrophomonas maltophilia OR02 *(S. maltophilia* 02*)* is a multidrug resistant bacterium that was isolated from East Fork Poplar Creek in Oak Ridge, Tennessee. *S. maltophilia* is a nosocomial opportunistic pathogen and is often seen in severely debilitated or immunosuppressed individuals and in the final stages of cystic fibrosis. It tolerates high levels of several metals including cadmium, chromium, copper, gold, lead, mercury, selenium, tellurium and uranium. It is also resistant to many antimicrobial agents such as carbapenems, aminoglycosides, cephalosporins, quinolones, penicillins and many β-lactam/β-lactamase inhibitor combinations. Transposon mutagenesis was used to identify an ampicillin sensitive mutant. Minimum inhibitory concentration testing was used to show that the mutant *S. maltophilia* AJ22 was sensitive to ampicillin. Gene rescue and DNA sequencing were used to obtain the DNA sequence and then the Basic Local Alignment Search Tool was used to determine that it was *ampG*. *ampG* is an inner membrane permease involved in transporting murein sacculus degradation products from the periplasm and into the cytosol.

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Chapter I: Introduction

1.1 Y-12 Plant and S-3 Ponds

During World War II, the Y-12 plant in Oak Ridge, Tennessee was responsible for processing uranium for nuclear bombs. When hydrogen bombs were being made during the Cold War, the plant used tons of mercury to process lithium. This caused mercury to be spilled into East Fork Poplar Creek and the surrounding area. Four S-3 ponds, located at the origin of the creek, were used to dispose of the acidic waste that had uranium and other heavy metal contaminants. Because the S-3 ponds were unlined, waste seeped into the creek (1). 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. These sites added aluminum, fluorine, potassium, sulfate, technetium-99 and plutonium-239. The waste stopped being dumped in the S-3 ponds in 1983, and the waste still in the ponds was treated by neutralization and biodenitrification. The ponds were then capped and now serve as a parking lot (2).

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

S. maltophilia 02 was isolated from East Fork Poplar Creek. This Gram negative strain grows in toxic levels of copper, zinc, platinum, mercury, gold, cadmium, lead, chromium and selenium salts (1). It is also resistant to 20 μg/ml chloramphenicol, 12 μg/ml tetracycline, 50 μg/ml kanamycin, 100 μg/ml streptomycin and 100 μg/ml ampicillin (Unpublished data).

1.3 Metal Resistance

Interestingly, *S. maltophilia* 02 is sensitive to silver, which acts as an antimicrobial agent by binding sulfur groups of proteins in bacterial cell walls and leads to cell death. Similar to the *S. maltophilia* 02 strain, *S. maltophilia* Sm777 also tolerates high levels of many toxic metals including cadmium, lead, cobalt, zinc, mercury, silver, selenium, tellurium and uranium (3).

1.4 *Stenotrophomonas maltophilia*

Stenotrophomonas maltophilia (*S. maltophilia*) is a multidrug resistant bacterium. It is a nosocomial opportunistic pathogen and is often seen in severely debilitated or immunosuppressed individuals and in the final stages of cystic fibrosis (4). There is a high mortality rate associated with *S. maltophilia* infections mainly because of its resistance to antimicrobials (5). The cells of *S. maltophilia* can be either straight or slightly curved and are non-sporulating bacilli with a length of 0.5 to 1.5 μm. It is motile with a few polar flagella and is an obligate aerobe that cannot grow at lower than 5°C or higher than 40°C. The optimal temperature for growth is 35°C and methionine or cysteine is required for growth for almost all strains (6). It is oxidase negative, however recently data has suggested that some isolates are oxidase positive. It produces acid from maltose but not from glucose which is a distinguishing feature setting it apart from *Pseudomonas aeruginosa* which produces acid from glucose (7).

S. maltophilia can be found inside and outside of hospitals and has been found in many water sources including rivers, wells, a hypereutrophic lake, bottled water and sewage. It has been isolated from several soil sources and plant rhizosphere environments

(6). It has also been found in biofilms on fractured surfaces in aquifers and it has the ability to adhere to plastics and form biofilms (7). It can form biofilms in showerheads (8) and potable water distribution systems which poses a risk of infection to immunocompromised people. It is also able to form biofilms on Teflon, glass and host tissues. Biofilms have been associated with 65% of nosocomial infections (7). Pili and fimbriae are used in the adhesion and biofilm formation (8). Patient to patient spread has not been seen, however small outbreaks have been caused by contaminated water sources. The isolates are usually very diverse both genotypically and phenotypically. Phylogenetic clustering has been seen, as about half of the clinical isolates have been found to be very similar (8).

S. maltophilia promotes plant growth and is antagonistic towards plant pathogens. For these reasons, it has been used in the development of biopesticides and is being studied for biological control of plant pathogens. It has the potential to be used for soil decontamination because it can degrade xenobiotic compounds and metabolize high molecular weight polycyclic aromatic hydrocarbons (3).

Several selective media have been developed to isolate *S. maltophilia*. One of these is *Xanthomonas maltophilia* selective medium, which was created for its isolation from soil and rhizosphere environments. This medium contains: bacitracin, cephalexin, neomycin, novobiocin, penicillin G, and tobramycin. It also contains two antifungal agents, nystatin and cycloheximide; and maltose and bromothymol blue (6).

Patients are more susceptible to a *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. *S. maltophilia* is commonly associated with pneumonia, and acute and chronic obstructive pulmonary disease, biliary sepsis, bacteremia, endophthalmitis, endocarditis, meningitis and obstructive lung cancer (7). It has also been present in infections of bones, joints, eyes, the urinary tract and soft tissues. Bacteremia, usually from an indwelling catheter, and pneumonia, from a ventilator, are the most common diseases that *S. maltophilia* causes (8). It also causes bacterial infections among cystic fibrosis patients (9) and colonization is found in the respiratory tract of about one-third of the patients (8).

1.5 Comparison of clinical and environmental strains

Clinical isolates have a higher rate of mutation when compared to environmental isolates. This suggests that the clinical isolates adapt to their environment. It has been proposed that the acquisition of antibiotic resistance genes occurs in the environment and that once a strain obtains access to a clinical environment, it retains the gene (7). A clinical strain, *S. maltophilia* K279A, was compared to an environmental strain, *S. maltophilia* R551-3. It was found that the genomes of both strains contained genes for the mismatch repair system, the nucleotide excision system, the guanine oxidation system, the recombination repair system and the SOS system. There were 41 genomic islands in K279a and 36 in R551-3. Whether the genomic islands are present or not represents a major source of the heterogeneity between the clinical and environmental strains. The gene products of the genomic islands appeared to play a role in interactions with the environment and included metal resistance genes, LPS genes, type I and IV secretion systems, and filamentous hemagglutinin genes (7).

1.6 Antibiotics

S. maltophilia is intrinsically resistant to many broad spectrum antimicrobial classes including aminoglycosides, cephalosporins, quinolones, carbapenems and penicillins (8).

1.6.1 Aminoglycosides

Aminoglycosides bind to prokaryotic ribosomes resulting in impaired bacterial protein synthesis (10). *S. maltophilia* shows a resistance to many aminoglycosides including newer members like isepamicin (6). Although gentamicin seems to be one of the most active aminoglycosides against *S. maltophilia* in some studies (11), it still has does not prove to be effective the majority of the time (12). *S. maltophilia* is naturally resistant to aminoglycosides because it contains a chromosomal AAC(6′)-Iz, which is an aminoglycoside acetyl-transferase (13). This causes a resistance to amikacin, netilmicin, sisomicin and tobramycin (14). When *aac(6′)-Iz*, is deleted, there is an increased susceptibility to 2-deoxystreptamine aminoglycoside antibiotics including gentamicin, neomycin, netilmicin, sisomicin and tobramycin. Resistance is subsequently restored in complemented mutants that contain the gene (7). It's outer membrane also has a thermo-dependent permeability to these antibiotics (13). Temperature can change the chemical composition of the lipopolysaccharide which can then alter the resistance level. When strains were grown at 30°C and 37°C, there was an increased sensitivity to aminoglycosides for the strains grown at 37°C. It was suggested that the increased temperature caused there to be more aminoglycoside binding sites available (7). As with carbapenems, previous use of aminoglycosides is a risk factor for an infection (15).

1.6.2 Quinolones

Quinolones directly inhibit DNA synthesis by targeting the DNA gyrase. DNA gyrase consists of two subunits, GyrA and GyrB. These break the strands of a DNA segment, pass another segment through the break and then reseal the break. This topoisomerization reaction leads to the introduction or removal of DNA supercoils (16).

Quinolones have shown some success in the treatment of *S. maltophilia,* however there has been an increasing resistance to this class (17). Newer quinolones, including clinafloxacin, sparfloxacin and trovafloxacin, seem to have more success against infections than some of the older ones (6). *S. maltophilia* has shown susceptibility to moxifloxacin. There is a higher resistance to ciprofloxacin, ofloxacin, sparfloxacin and gatifloxacin from isolates that were obtained from cystic fibrosis patients than from non-cystic fibrosis patients. Levofloxacin could potentially be used as an aerosolized antibiotic for cystic fibrosis infections. Quinolones were effective at reducing the adherence and biofilm formation of *S. maltophilia.* Ciprofloxacin, grepafloxacin, moxifloxacin, norfloxacin, ofloxacin and rufloxacin were all effective in reducing the biofilm mass and moxifloxacin was the most successful at preventing adherence and reducing a preformed biofilm biomass. An intrinsic resistance to quinolones is present due to the Sm*qnr* gene (7). A stronger resistance to fluoroquinolones may develop through the selection of mutants with increased expression of SmQnr proteins or efflux pumps (SmeDEF or SmeVWX) (13). The SmeDEF efflux pump also contributes to quinolone resistance (7). When SmeDEF is over expressed there is a hyper-resistance to fluoroquinolones (8) because the SmeDEF pump contributes to the removal of some quinolones (13).

1.6.3 TMP-SMX

Trimethoprim and sulfamethoxazole both target steps in the folate biosynthesis pathway. Sulfamethoxazole inhibits the dihydropteroate synthase FolP, which then catalyzes the addition of dihydropterin diphosphate to *p*-aminobenzoic acid. Trimethoprim targets dihydrofolate reductase Dhfr, which is produced in a later step (18).

A study done on *S. maltophilia* strains showed that no strain in the study was resistant to both trimethoprim and sulfamethoxazole. A trimethoprim-sulfamethoxazole (TMP-SMX) combination has proved to be successful on these strains (19). Although TMP-SMX is the recommended drug for treatment, there is an in increasing resistance to this antibiotic combination (12). *In vitro* studies have revealed that TMP-SMX combined with either ciprofloxacin or tobramycin is more effective than just TMP-SMX (20). TMP-SMX has also been combined with ticarcillin-clavulanate or fluoroquinolones and synergy with these combinations has been observed in vitro for more than 50% of isolates (13). TMP-SMX has also been successfully combined with carbenicillin and rifampin (7).

1.6.4 Others Antibiotics

S. maltophilia is also resistant to macrolides, chloramphenicol, tetracyclines and polymyxins (7). Since there has been so little success with monotherapy, combinations of antimicrobial agents have been looked at (6). A combination of doxycycline and aerosolized colistin was successful in treating *S. maltophilia* pneumonia when TMP-SMX didn't work. Older antibiotics have also shown some success with rifampin treating an infection in combination with gentamicin and carbenicillin (7). Most strains are not resistant to the oxa-β-lactam moxalactam. However, there are hematologic side effects so it is not used in clinical infections (6). Like other gram-negative bacilli, it is also weakly

susceptible to erythromycin. Not only does it have a reduced permeability to erythromycin, but it can also pump the drug out through a multidrug efflux determinant (4). Multidrug resistant *S. maltophilia* have a higher level of biofilm formation than non-MDR isolates. The formation of a biofilm also has a correlation with the resistance to aztreonam, cefepime, ceftazidime, gentamicin, piperacillin-tazobactam and ticarcillin-clavulanic acid. There was no correlation between the biofilm formation and resistance to ciprofloxacin, levofloxacin, meropenem (7).

1.6.5 Carbapenems

Carbapenems are β-lactams and are generally used if a patient hasn't responded to any other antibiotics, however, they are becoming less effective because of the rise in multidrug-resistant bacteria. Carbapenems work by inhibiting cell wall synthesis and usually enter the bacteria through outer membrane proteins and acylate the penicillin-binding-proteins (PBPs). They can inhibit the peptidase domain of PBPs and peptide crosslinking. Once the PBPs are inhibited, autolysis continues; the peptidoglycan weakens and the osmotic pressure will cause the cell to burst (21).

S. maltophilia has exhibited resistance to many carbapenems and can hydrolyze them. This includes meropenem, imipenem and even biapenem, which is a newer member of this class. One study showed that three strains that were resistant to imipenem had a susceptibility to meropenem (6). Another study tested 80 isolates for imipenem and 70 isolates for meropenem and 100% of them showed a resistance to the antibiotics (11). Contributing to the intrinsic resistance of carbapenems is the presence of the inducible L1 carbapenemase (22). One study showed that the loss of L1 greatly increased the

susceptibility of *S. maltophilia* to imipenem, meropenem and panipenem (23). Previous use of carbapenems is also a risk factor for a *S. maltophilia* infection (15).

1.6.6 Cephalosporins

Cephalosporins bind to the bacterial PBPs and interrupt the formation of the peptidoglycan (24). Cephalosporin antibiotics seem to almost always be ineffective against *S. maltophilia* (13). L2 is a clavulanic acid-sensitive cephalosporinase (7) and demonstrates hydrolytic activity towards cephalosporins (7). L1 can also hydrolyze cephalosporins (12). Some *S. maltophilia* strains show a susceptibility towards ceftazidime (6). One study used newer-generation cephalosporins and showed that 37% of isolates were resistant to ceftazidime and 58% were resistant to cefepime. Cystic fibrosis strains were more resistant cefotaxime and cefepime than non-cystic fibrosis strains (7).

1.6.7 Penicillins

Penicillins are another class of antibiotic that inhibit the bacteria cell wall synthesis and also interact with penicillin-binding-proteins (PBPs) eventually causing lysis (25). Similar to cephalosporins, penicillins almost always exhibit poor activity against *S. maltophilia* (6). This is because penicillins can be hydrolyzed by both L1 and L2 (26). The loss of L1 or L2 does not affect penicillin G, ampicillin, amoxicillin or cloxacillin. The loss of L2 slightly decreases the resistance to carbenicillin and ticarcillin while the loss of L1 slightly decreases the resistance to piperacillin (23).

1.6.8 β-lactam/β-lactamase inhibitor combinations

There are six β-lactam/β-lactamase inhibitor combinations: amoxicillin-clavulanic acid, ampicillin-sulbactam, cefoperazone-sulbactam, piperacillin-tazobactam, ticarcillin-

clavulanic acid and sultamicillin (a mutual prodrug between ampicillin and sulbactam). Clavulanic acid and sulbactam are inhibitors of class A β-lactamases such as L2 (27). Ticarcillin-clavulanate has shown some success against *S. maltophilia* infections while the piperacillin-tazobactam, amoxicillin-clavulanic acid and ampicillin-sulbactam combinations have yielded poor results. Cefoperazone-sulbactam has only been successful on patients with *S. maltophilia* pneumonia (6). Resistance increased in isolates collected between 1994 and 1997 to ticarcillin-clavulanate but this did not correspond to antimicrobial use since the usage actually decreased in that time period. This resistance may have resulted from increased use of parenteral amoxicillin, amoxicillin-clavulanate, ticarcillin and piperacillin-tazobactam (7).

1.7 Causes for Resistance

One mechanism for resistance to β-lactams is low membrane permeability. Chromosomally encoded multidrug resistance efflux pumps, β-lactamases and antibiotic-modifying enzymes all have a role in *S. maltophilia's* intrinsic antibiotic resistance. It has been suggested that *S. maltophilia* uses its metabolic machinery to detoxify and break down harmful compounds such as antibiotics, which could then be used as a nutrient source. Molecular mechanisms that contribute to its resistance are acquired by horizontal transfer through plasmids, biofilms, transposons, integrons, integron-like elements and insertion element common region elements. (7).

S. maltophilia K279a contains β-lactamases, L1 and L2. It also has aminoglycoside modifying enzymes APH 3´II and AAC 6´I that cause resistance to all aminoglycosides except gentamicin (8). In a study done on 118 isolates, when L1 was present 100% of the isolates were resistant to imipenem and 92.3% were resistant to

meropenem. When L2 was present, 100% of the isolates were resistant to imipenem and 91.1% were resistant to meropenem (12).

Multidrug resistance is usually mediated by the over-production of resistance-nodulation division (RND) type efflux pumps (8). Multidrug efflux pumps are made up of a membrane fusion protein, an energy dependent transporter and an outer membrane protein (7). Two RND efflux systems have been found, SmeABC and SmeDEF (3). The *Stenotrophomonas* multiple-efflux (*sme*) *smeDEF* operon encodes a multidrug efflux pump. The SmeDEF efflux pump is one of the reasons *S. maltophilia* is resistant to β-lactams, aminoglycosides, chloramphenicol, erythromycin, quinolones and tetracycline (7). Over expression of SmeDEF in *S. maltophilia* K279a causes hyper-resistance to fluoroquinolones, chloramphenicol and tetracycline. This hyper-expression can occur because of loss-of function mutations in the TetR-type transcriptional repressor, *smeT* or through other unidentified mutations (8). Integrons have an integrase-encoding gene that allows for the insertion of antibiotic resistance gene cassettes between highly conserved nucleotide sequences (7). In Argentina and Taiwan, strains have been found with class 1 integrons. This indicates that the integrons have a role in the TMP-SMX resistance through the *sul*1 gene that is carried as part of the 3′ end of the class 1 integron (20). *S. maltophilia* also encodes antibiotic-inactivating enzymes such as metallo-beta-lactamases, cephalosporinases and aminoglycoside-modifying enzymes (4).

1.8 β-lactamase

The resistance to β-lactams is primarily intrinsic and is mediated by L1 and L2 (9). These are chromosomally encoded *β*-lactamases that are inducible. L1 is produced by

all wild-type strains and it belongs to the metalloenzyme family (6). It is a Zn^{2+}-dependent metalloenzyme and it hydrolyzes all of the β-lactam classes (penicillins, cephalosporins and carbapenems) except monobactams (9). L1 is a holoenzyme and consists of a tetramer of four equal subunits. It does not have a susceptibility to β-lactamase inhibitors like clavulanate (6) but it is inhibited by aztreonam (11). In its native state, L2 exists as a dimer (6) and contains a serine active-site (9) and clavulanic acid-sensitive cephalosporinase (7). It also hydrolyzes aztreonam and it is susceptible to β-lactamase inhibitors (6). L1 and L2 use different export systems for periplasmic translocation. The L1 β-lactamase uses a Sec export system while the L2 β-lactamase uses a Tat export system. Clinical isolates that were exposed to imipenem, cefoxitin or ampicillin demonstrate heterogeneity for β-lactamase induction. *AmpR, ampC, ampN and ampD* genes are all needed for lactamase expression. L1 and L2 are differentially regulated. Their expression is controlled at the transcription level by the *ampR* gene which is found upstream of L2 as part of an *ampR*-L2 module. The basal-level expression of L1 requires AmpR and it is also needed for the induced expression of both L1 and L2 (7). The AmpR-β-lactamase module has a divergently transcribed control unit which is where the AmpR protein regulates the β-lactamase gene expression. There are two types of β-lactamase genes in these modules, *ampC*-like and class A β-lactamase genes. L2 is an *ampR*-linked class A β-lactamase gene, and L1 is an *ampR*-unlinked class B β-lactamase gene (28). AmpR is a transcriptional regulator of the expression of *ampC,* which has a role in the recycling of bacterial cell wall components. When AmpR is bound with anhydro-*N*-acetylmuramyl peptide, AmpC expression is activated. When AmpR is bound with UDP-*N*-acetylmuramic acid-pentapeptide, the expression of AmpC is

repressed. AmpG is also used in cell wall recycling. It helps in the transport of degraded cell wall components into the cytoplasm. AmpD is associated with the cleavage of the components into 1,6-anhydromuramic acid and peptide (7). The AmpD protein degrades the AmpR activator ligand. This represses the production of AmpC during normal growth and the loss of AmpD then derepresses the production of AmpC. This causes a strong β-lactam resistance (29). Also needed for the expression of L1 and L2 is an *ampN-ampG* operon. When the *ampN* gene is disrupted, it causes a polar effect on the expression of *ampG* (7). Penicillin-binding proteins (PBPs) are membrane bound enzymes that have a role in the last stages of peptidoglycan biosynthesis. When a mutation or β-lactamase saturation occurs and causes a PBP to lose its biological activity in peptidoglycan synthesis, there is a change in the peptidoglycan structure. This disturbs the balance of the degraded peptidoglycan components in the cytosol which leads to an induction of the chromosomal β-lactamase gene which is necessary for the resistance to β-lactams (29). PBPs are inhibited by the β-lactam, imipenem (5).

1.9 Transposon Mutagenesis

Transposon mutagenesis was used to identify an ampicillin resistance gene in the *Stenotrophomonas maltophilia* OR02 strain. The EZ-Tn*5*™ <*R6Kγori*/KAN-2>Tnp Transposome™ is a DNA/transposon protein complex (30). 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 incorporated itself randomly into this bacterium's genome. The resulting colonies on LB agar plates supplemented with 800 μg/ml kanamycin contain transposon inserts. Screening by replica plating on LB-agar plates

containing and lacking 100 ampicillin μg/ml identified a mutant, AJ22, which was sensitive to ampicillin. The interrupted gene can be identified by performing DNA sequencing.

Chapter II: Hypothesis

Transposon mutagenesis was used to identify a mutant of *Stenotrophomonas maltophilia* OR02 that was sensitive to ampicillin. By sequencing this mutant, we expected to identify a gene that encoded β-lactamase or a gene that encoded a β-lactamase-regulator. Since there are potentially two β-lactamase genes in *S. maltophilia*, identifying a gene for β-lactamase-regulator was more likely. Unsurprisingly, I identified a putative *ampG* gene, which encodes a protein for β-lactamase-regulation.

Chapter III: Methods

3.1 Bacterial Strains

Stenotrophomonas maltophilia 02 (ATCC #53510) was purchased from the American Type Culture Collection (Manassas, VA). *Escherichia coli* (*E. coli*) strain ECD100D *pir116* was purchased from Epicentre (Madison, WI).

3.2 Growth Media

Genomic Grade™ Culture Media LB (Lennox) Broth was obtained from Growcells.com (Irvine, CA) subsidiary of Molecular Biologicals International, Inc. It consisted of 10 g/l tryptone, 5 g/l yeast extract and 5 g/l sodium chloride. When required, LB agar was supplemented with 1.6% agar Amresco (Solon, Ohio). The AJ22 *S. maltophilia* 02 mutant was grown in the presence of 800 μg/ml kanamycin Amresco (Solon, Ohio), and *E. coli* containing recombinant transposon plasmids were grown in the presence of 50 μg/ml kanamycin. Wild type *S. maltophilia* 02 was grown in the absence of kanamycin.

3.3 Minimum Inhibitory Concentration (MIC) Testing

Overnight cultures of *S. maltophilia* O2 and AJ22 were diluted 1/50 into fresh LB medium and mixed with 0, 100, 200, 300, 400, 500, 600, 700 or 1000 μg/ml Ampicillin (Thermo Fisher Scientific, Waltham, MA). After measuring the turbidity using a Klett™ Colorimeter (Fisher Scientific, Waltham, MA), they were incubated for 24 hours overnight at 30°C in a roller drum (Fisher Scientific, Waltham, MA). Turbidity was measured again. This experiment was repeated four times in order to obtain averages with

standard deviations. The averages were used to determine standard deviation using the STDEV function in Excel. TINV function was used to calculate the inverse of the two-tailed T Distribution using TINV(probability, degrees_freedom). Standard error was calculated using the following equation for the student T-test (31):

$$\mathbf{Error} = \frac{\boldsymbol{t}_{(\mathbf{95\%CI},(\mathbf{N-1})\mathbf{d.f.})} * \boldsymbol{StdDev}}{\sqrt{\boldsymbol{N-1}}}$$

Where N is the Number of observation, CI is the confidence interval, d.f is the degrees of freedom and Std Dev is the standard deviation.

3.4 Transposon Mutagenesis

Transposon mutagenesis (30) was used to generate an ampicillin sensitive mutant of *Stenotrophomonas maltophilia* OR02. The EZ-Tn*5* <R6Kγori/KAN-2>Tnp Transposome (Epicentre, Madison, WI) was used to electroporate electrocompetent cells. The cells were prepared by growing them at 30°C to an optical density (600 nm) between 0.6 and 1.0. The cells were cooled on ice in two 50 ml centrifuge tubes and pelleted at 4°C and 7,000 x g for 5 minutes. The supernatant was poured off, the cells were resuspended in 15 ml of sterile ice cold water and then water was added to 50 ml. The cells were pelleted, resuspended again in 15 ml of sterile ice cold water and water was added to 50 ml. The supernatant was poured off and the cells were resuspended in 200 µl of ice cold water. 40 µl of the cells were added to sterile 1.7 ml tubes. 0.5 µl of EZ-Tn*5* transposome was added to the tubes and mixed. The cell/transposome mixture was put in an ice-cold electroporation cuvette with a 2 mm gap and the mixture was tapped to the bottom of the tube. The cells were pulsed at 25 µF, 200 ohms and 2.5 kV. 960 µl of SOC

medium (0.5% (w/v) Yeast Extract (Amresco, Solon, OH), 2% (w/v) Tryptone (Amresco, Solon, OH), 10 mM NaCl (Amresco, Solon, OH), 2.5 mM KCl (Amresco, Solon, OH), 10 mM $MgCl_2$, 20 mM $MgSO_4$ (Thermo Fisher Scientific, Waltham, MA), and 20 mM Glucose (Amresco, Solon, OH)) was immediately added and mixed by pipetting to recover the cells. The cells were then transferred to a 1.5 ml tube and incubated at 30°C in a shaker for 45-60 minutes. The cells were then spread on LB plates containing 800 μg/ml kanamycin. The colonies that grew contained the transposon insert. These colonies were gridded on a grid on kanamycin plates, incubated overnight at 30°C and then screened for mutants by replica plating (30). A replica plating block (Bel-Art, Wayne, NJ) was used to transfer the cells from the kanamycin plates to the 100 μg/ml ampicillin plates. The replica plating block was covered with a sterilized velvet cloth, (Bel-Art, Wayne, NJ) and the gridded plate was placed on block to transfer the transformed bacteria to velvet. Once the plate was removed, the bacteria were transferred to a fresh kanamycin plate, plate number 1. The velvet was discarded and a new velvet was placed on the block. The bacteria were then transferred from plate 1 onto the new velvet. This velvet was then used to transfer the bacteria to the ampicillin and final control kanamycin plate. These plates were incubated overnight at 30°C and growth or absence of growth was recorded. If a colony grew on the kanamycin plate but not on the ampicillin plate, it was considered to be an ampicillin sensitive mutant.

3.5 Genomic DNA Purification

Genomic DNA was purified using a Promega (Madison, WI) Wizard® Genomic DNA Purification Kit. 1 ml of overnight culture was pelleted by centrifugation for 2 minutes at 13,000 x g and the supernatant was discarded. The cells were then lysed by

adding 600 µl of Nuclei Lysis Solution and mixing gently by pipetting. This mixture was incubated for 5 minutes at 80°C and cooled to room temperature. Next, 3 µl of RNase Solution was added, mixed, incubated at 37°C for 15 minutes and cooled to room temperature. 200 µl of Protein Precipitation Solution was added and then vortexed for 20 seconds. The mixture was incubated on ice for 5 minutes and centrifuged at 13,000 x g for 3 minutes. The DNA was precipitated by transferring the supernatant to a clean tube that contained 600 µl of room temperature isopropanol and then mixing. This was centrifuged at 13,000 x g for 2 minutes and the supernatant was discarded. The ethanol was aspirated and the pellet was air-dried for 10-15 minutes. The DNA pellet was then rehydrated by adding 100 µl of Rehydration Solution and incubating it overnight at 4°C.

3.6 Partial Digestion with *Bfu*CI

The purified DNA was partially digested with *Bfu*CI. A reaction containing 2 µl of New England BioLabs (Ipswich, MA) 10X CutSmart Buffer, 4.9 µl nuclease free water, 0.1 µl of diluted (1:200) *Bfu*CI and 13 µl of purified DNA was set up on ice. It was incubated at 37°C for 25 minutes in a thermocycler to digest the DNA, followed by 80°C for 20 minutes in a thermocycler to inactivate the *Bfu*CI.

3.7 *Kpn*I and *Pvu*II Digestions

The purified genomic DNA was also digested using the restriction enzymes, *Kpn*I and *Pvu*II. For 20 µl digestions, 2 µl of 10X CutSmart Buffer, 5 µl Nuclease Free Water and 1 µl of the enzyme was added to 12 µl of the genomic DNA. This mixture was then incubated for 60 minutes at 37°C.

3.8 Gel Electrophoresis

A 1% gel was prepared by microwaving 1.3g of BioExcell® Agarose LE (Worldwide Medical Products, Bristol, PA) in 130 ml of 1X TBE (Amresco, Solon, OH). 13 µl of GelGreen Nucleic Acid Stain (Embi Tec, San Diego, CA) was then mixed in using a stir bar. This was poured into the tray and combs were inserted to form wells. The gel was placed into a RunOne™ Electrophoresis System (San Diego, CA). Amresco (Solon, OH) 10X TBE buffer was diluted to 1X containing 0.089 M Borate and 0.002 M EDTA and was poured over the gel. The gel was loaded by mixing 2 µl of Amresco Agarose Gel Loading Dye, 6X with 3 µl of each digested and undigested sample and loading each one into a well. 3 µl of an Amresco 1 kb DNA ladder was also loaded with 2 µl of the loading dye. 100 volts of current was then used to separate the DNA. Once the gel was finishing running a picture was taken using an Embi Tec PrepOne™ Sapphire (San Diego, CA).

3.9 T4 DNA Ligation

The digestions were heated at 80°C for 20 minutes. A ligation was then performed by adding 10 µl 10X T4 DNA Ligase Buffer, 74 µl Nuclease Free Water and 2 µl T4 DNA Ligase (New England BioLabs, Ipswich, MA) to 14 µl of the digested DNA. This mixture was incubated at 4°C overnight. Each ligation was then precipitated with 10 µl of 3M sodium acetate and 200 µl of 95% ethanol and incubated at -20°C for 10 minutes. They were pelleted by centrifugation for 10 minutes. The supernatant was poured off and the pellet was washed with 200 µl of 70% ethanol and gently inverted. This was centrifuged again and then dried in the CentriVap (Labconco, Kansas City,

MO). Each pellet was resuspended in 10 µl Nuclease Free Water and mixed with 100 µl of ECD100D *pir116*, calcium chloride, competent, *E. coli* cells.

3.10 Preparation of *pir*116 competent cells

5 ml of *pir*116 overnight culture was added to 100 ml LB. These cells were grown at 37°C in a shaker until they reached the optical density (600 nm) of 1.0 which was measured using a BioPhotometer (Eppendorf, Hauppauge, NY). They were then cooled on ice, transferred to 50 ml tubes and centrifuged at 4°C and 5,000 x g for 5 minutes. The supernatant was poured off, the cells were resuspended in 15ml of sterile 0.15 M NaCl and pelleted again at 4°C and 5,000 x g for 5 minutes. The supernatant was poured off and the cells were resuspended in ice cold 1 ml of transformation buffer (15% glycerol, 0.1 M $CaCl_2$, 10 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$). 400 µl of the resuspended cells were pipetted into 1.5 ml tubes and incubated overnight on ice in the refrigerator. The cells were then frozen at -80°C to make them competent.

3.11 Transformation into *pir*116 competent cells

ECD100D *pir*116 competent cells were thawed on ice. 100 µl of the cells were added to each sample and then incubated on ice for 30 minutes. They were heat shocked in a 42°C water bath for 50 seconds and then placed on ice. 900 µl LB was added to each sample and then incubated in a shaker at 37°C for 60 minutes. The cells were then plated on kanamycin plates and incubated at 37°C overnight.

3.12 Plasmid Prep

Plasmid DNA was purified using a Promega Wizard® Plus SV Minipreps DNA Purification System (Madison, WI). A 5 ml culture was pelleted by centrifugation at 7,000 x g for 5 minutes. The supernatant was poured off and the pellet was resuspended using 250 µl of Cell Resuspension Solution. 250 µl of Cell Lysis Solution was added to each sample and inverted 4 times to mix. 10 µl of Alkaline Protease Solution was added, inverted 4 times to mix and then incubated at room temperature for 5 minutes. 350 µl of Neutralization Solution was added and then inverted 4 times and centrifuged at room temperature for 10 minutes. A spin column was inserted into a collection tube and the cleared lysate was decanted into the spin column. This was centrifuged at top speed for 1 minute at room temperature. The flow-through was discarded and the spin column was reinserted into the collection tube. 750 µl of Wash Solution with ethanol was added, centrifuged at top speed for 1 minute and then the flow-through was discarded. This was repeated with 250 µl of Wash Solution, centrifuged for 2 minutes and the flow-through was discarded. The spin column was then inserted into a sterile 1.5 ml microcentrifuge tube and 100 µl of Nuclease Free Water was added to the spin column and centrifuged at top speed for 1 minute. The spin column was discarded and the DNA was stored at -20°C.

3.13 DNA Sequencing

The GenomeLab™ Dye Terminator Cycle Sequencing with Quick Start Kit (Brea, CA) was used for DNA sequencing. A NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to determine the amount of DNA to use in each

sequencing reaction. 2 µl of DNA was placed on the NanoDrop, and it measured the concentration. This was used to calculate the amount of DNA and water that would be added to a GeneMate (Lodi, CA) 0.2 ml PCR tube to make a 10 µl mix. This was incubated in the thermocycler at 96°C for 1 min. 2 µl of a primer (Table 1) and 8 µl DTCS Quick Start Master Mix were added. These mixes were then incubated in the thermocycler (Eppendorf, Hauppauge, NY) using the following program: 90°C for 20 seconds, 50°C for 20 seconds, 60°C for 4 minutes for 30 cycles and then held at 4°C.

3.13.1 Sequencing Reaction Cleanup

A 0.5 ml microfuge tube with 60 µl of cold 95% ethanol was prepared for each sample and then placed on ice. Fresh Stop Solution/Glycogen mixture was prepared using the following for each sample: 1.2 M Sodium Acetate (pH 5.2), 40 mM Na_2-EDTA (pH 8.0) and 8 mg/mL glycogen. The sequencing reactions were then transferred to the tubes containing the 95% cold ethanol and immediately centrifuged at 14,000 rpm at 4°C for 15 minutes. The supernatant was removed and the pellet was washed twice with 200 µl of ice cold 70% ethanol. They were centrifuged immediately after each rinse at 14,000 rpm at 4°C for 2 minutes. The ethanol was pipetted off and the DNA pellet was dried using a CentriVap (Labconco Corporation, Kansas City, MO). The pellets were resuspended in 40 µl of Sample Loading Solution. It was then analyzed with the Beckman Coulter CEQ 2000XL DNA analysis system (Fullerton, CA).

3.14 Sequence Analysis

The sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) which compares the sequence to others in the database. Nucleotide blast

(blastn) was used to find matching sequences. Gene Studio was used to assemble overlapping sequences and correct any ambiguities in order to create a consensus contig. The sequence was then translated to a protein sequence using ExPASy (Expert Protein Analysis System) (32) and Genome Compiler (Genome Compiler Corporation, Los Altos, CA). This sequence was further analyzed using blastp (protein blast) and several homologs were identified. These were downloaded from BLAST (33), aligned using ClustalX (34) and then viewed using GeneDoc. A phylogenetic tree was constructed in MEGA 7.0 (35) using the Maximum Likelihood method.

3.15 Probe Labeling

PCR was used to amplify a part of the EZ-Tn*5* transposon. The reaction without biotin contained: 25 µl of 2X GoTaq DNA Polymerase (Promega, Madison, WI), 6.25 µl of 4 µM Kan Probe F (Table 1), 6.25 µl of 4 µM Kan Probe R (Table 1), 11.50 µl of nuclease free water and 1 µl of DNA. The reaction with biotin contained: 25 µl of 2X GoTaq DNA Polymerase, 6.25 µl of Kan Probe F (4 µM), 6.25 µl of Kan Probe R (4 µM), 6.5 µl of nuclease free water, 2.5 of µl Biotin-dUTP (1 mM) (PromoKine, Heidelberg, Germany), 2.5 µl of Biotin-dCTP (1 mM) and 1 µl of DNA. The DNA was recombinant plasmid containing the Tn*5* transposon. The PCR reactions were carried out using a thermocycler with the following program: 95°C for 2 minutes, then 35 cycles of 95°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute, followed by 72°C for 10 minutes and holding at 10°C.

3.16 PCR Purification

A QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) was used. 5 volumes of the Buffer PB to 1 volume of the PCR reaction was added and mixed. A MinElute column was placed in a 2 ml collection tube and the sample was added. This was centrifuged for 1 minute and the flowthrough was discarded and the MinElute column was placed back into the collection tube. 750 µl of Buffer PE was added to the MinElute column centrifuged for 1 minute and the flowthrough was discarded and the column was placed back in the collection tube. The column was centrifuged in a 2 ml collection tube for 1 minute to remove residual ethanol. The MinElute column was then placed in a clean 1.5 ml microcentrifuge tube. The DNA was eluted by adding 10 µl of Buffer EB to the center of the MinElute membrane, letting it stand for 1 minute and centrifuging the column for 1 minute.

3.17 Southern Blotting

Southern Blotting was performed using a Chemoluminescent Nucleic Acid Detection Module (Thermo Fisher Scientific, Waltham, MA) to determine if the Tn*5* Transposon inserted more than once in the AJ22 genome. Genomic DNA was digested with *Kpn*I and *Pvu*II and then gel electrophoresis was performed. The gel was placed in ethidium bromide for 30 minutes and then a picture was taken. The gel was briefly rinsed in distilled water and then shaken in 50 ml of Amresco Depurination solution (containing 0.25 M HCl) for 30 minutes to remove the adenine and guanine bases and to break the DNA into smaller pieces to facilitate the transfer. The Depurination solution was poured off, the gel was rinsed with distilled water and shaken in 50 ml of Amresco Denaturation

solution (1.5 M NaCl and 0.5 M NaOH) for 20 minutes. The Denaturation solution was poured off, the gel was rinsed with distilled water and shaken in 50 ml of Amresco Neutralization solution (1.5 M NaCl and 1 M Tris, pH 7) for 20 minutes. The Neutralization solution was poured off and another 50 ml was added for 20 minutes.

For capillary transfer, SSC Buffer (saline sodium citrate), 20X Liquid Concentrate (Amresco, Solon, OH) containing 3 M NaCl and 300 mM sodium citrate, was added to a plastic container. A wick slightly wider than the gel was made from filter paper (Fisher Scientific, Pittsburgh, PA) and was draped over a box placed in the 20X SSC so each end was submerged in the 20X SSC Buffer. The gel was placed upside down on top of the wick and air bubbles were removed. A piece of Biodyne® B Pre-Cut Modified Nylon Membrane, 0.45 μm (Thermo Fisher Scientific, Waltham, MA) was soaked in 20X SSC and then placed on top of the gel and air bubbles were removed. Three pieces of filter paper were placed on top of the membrane. Six inches of paper towels, cut to the same size as the gel, were placed on top of the filter paper, weighted down and left overnight. The membrane was then baked in a Mini Hybridization Oven (Bellco Glass, Inc., Vineland, NJ) at 80°C for 30 minutes to fix the DNA to the membrane.

3.18 Hybridization and Detection

A North2South® Chemiluminescent Hybridization and Detection Kit (Thermo Fisher Scientific, Waltham, MA) was used. The North2South® Hybridization Buffer was equilibrated to room temperature. The blot was placed in a 50 ml tube and 0.1 ml per cm^2 of Hybridization Buffer was added to completely cover the membrane. The container was then placed in the Mini Hybridization Oven and rotated at 55°C for 30 minutes. While

pre-hybridizing, the biotinylated probe was denatured. The probe was heated at 98°C for 10 minutes and then placed on ice for 5 minutes. After pre-hybridization, about 30 ng of probe per ml of hybridization buffer was added to the container. This was incubated overnight with rotation at 55°C in the Mini Hybridization Oven.

The Hybridization Buffer was poured off and the North2South® Hybridization Stringency Wash Buffer (2X) was equilibrated to room temperature for the stringency washes. Once the wash buffer was fully in solution, an equal volume of sterile ultrapure water was added. The 1X buffer contained 2X SSC/0.1% SDS. The blot was washed three times for 15-20 minutes per wash at 55°C with rotation. 0.2 ml of 1X Stringency Wash Buffer per cm^2 of membrane was added.

The Stringency Wash Buffer was decanted and the membrane was transferred to a tray using clean forceps. Enough Blocking Buffer was added to sufficiently cover the membrane, using at least 0.25 ml/cm^2, and it was shaken for 15 minutes at room temperature using a VWR S-500 Orbital Shaker (Radnor, PA) and then poured off. 66 µl of Streptavidin-HRP was added to 10 ml of 1X Blocking Buffer, poured over the blot and shaken for 15 minutes at room temperature. Wash Buffer (4X) was diluted to 1X with sterile ultrapure water. The membrane was washed 4 times for 5 minutes each with the 1X Wash Buffer and shaken at room temperature. The membrane was then placed in a clean tray, 0.25 ml/cm^2 of Substrate Equilibrium Buffer was added, and the membrane was shaken for 5 minutes at room temperature.

A Substrate Working Solution was prepared by mixing equal volumes of Luminol/Enhancer Solution and Stable Peroxide Solution. Enough of this solution was

prepared to completely cover the membrane using about 0.1 ml/cm^2. The membrane was incubated with the Substrate Working Solution on top of it for 5 minutes at room temperature. The substrate was then drained, the membrane was transferred to plastic wrap and all air bubbles were removed. A picture of the membrane was taken using the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Hercules, CA).

Table 1. Primers used for sequencing

Primer	Sequence
AJ22 F1	5’ – ATC CCG GTG GTG ATC CT – 3’
AJ22 F2	5’ – GGC CAT GAT CTC GTA GGA AAC – 3’
AJ22 F3	5’ – TCG CTG TTG TCA GCA CTT TC – 3’
AJ22 F4	5’ – ATG CTT GGC GCA TTG AAA G – 3’
AJ22 F5	5’ – TGT TCA GCA GTG AAC TGG A – 3’
AJ22 F6	5’ – TTG CCA TCG CCC AAC TG – 3’
AJ22 R1	5’ – TCG CTG TTG TCA GCA CTT T – 3’
AJ22 R2	5’ – GCA GGT TTC CTA CGA GAT CAT – 3’
AJ22 R3	5’ – CAG CAG TGA ACT GGA GGT T – 3’
AJ22 R4	5’ – TCG CTG TTG TCA GCA CTT TC – 3’
AJ22 R5	5’ – TGG ACG AAA GCC TGC TC – 3’
AJ22 R6	5’ – GGT GAT GAA CTG GTA GTC GAT – 3’
KAN-2 FP-1	5’ – ACC TAC AAC AAA GCT CTC ATC AAC C – 3’
R6KAN-2 RP-1	5’ – CTA CCC TGT GGA ACA CCT ACA TCT – 3’
Kan Probe F	5’ – GGT ATA AAT GGG CTC GCG ATA A – 3’
Kan Probe R	5’ – CCG ACT CGT CCA ACA TCA ATA C – 3’
AJ22_AmpN_R1	5’ – GCC ATC CGG TCG GAA CA – 3’
AJ22_AmpN_R2	5’ – TCG CGG TAG AAG CAG TGA – 3’

Chapter IV: Results

4.1 Transposon Mutagenesis

Transposon mutagenesis was used to identify ampicillin resistance genes in *S. maltophilia* (30). The EZ-Tn*5* transposome was electroporated into this strain and transformants were identified by plating the electroporated cells on LB-agar plates containing 800 µg/ml kanamycin. It was necessary to use this high concentration because this strain is already resistant to 50 µg/ml kanamycin. This high concentration allowed transformants to grow at a faster rate than the untransformed cells. After gridding 1760 transformants onto a LB-kan plate containing 800 µg/ml kanamycin and growing them overnight, they were replica plated onto 100 µl/ml ampicillin plates (Figure 1). Transformant, AJ22, grew on the LB-kan plate but not on the ampicillin plate and was therefore identified as an ampicillin sensitive mutant.

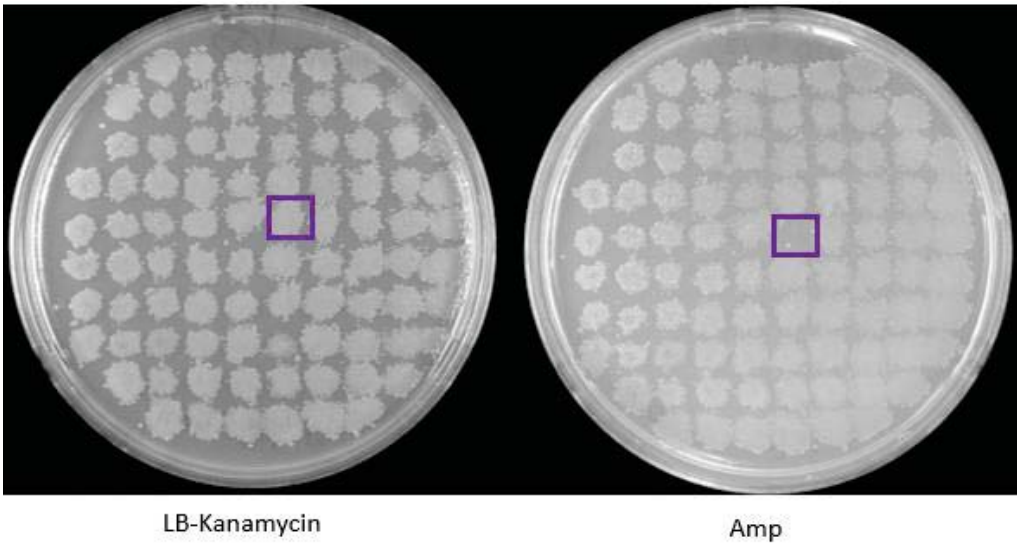


Figure 1: Replica plating of *S. maltophilia* on kanamycin and ampicillin plates. Plate A is a kanamycin plate and the colony in the box grew. The corresponding colony did not grow on Plate B which is an ampicillin plate. That strain is an ampicillin sensitive mutant and was named *S. maltophilia* 02 AJ22.

4.2 Minimum Inhibitory Concentration Testing

Minimum Inhibitory Concentration (MIC) testing was then performed on *S. maltophilia* and AJ22 to compare the ampicillin resistance. Nine concentrations of ampicillin (0, 100, 200, 300, 400, 500, 600, 700 and 1000 μg/ml) were used. The turbidity was measured in Klett units before and after they were incubated overnight at 30°C. The difference between the 0 hour and 24 hour readings were averaged for the four trials and error was calculated using a t-test with a 95% confidence level. The *S. maltophilia* 02 strain was resistant to ampicillin even at 1000 μg/ml as shown in Figure 2. However, AJ22's turbidity dropped at each increasing ampicillin concentration until 400 μg/ml where it leveled off and only increased about 7-10 klett for 500-1000 μg/ml. This showed that AJ22 is sensitive to ampicillin and the MIC is 400 μg/ml.

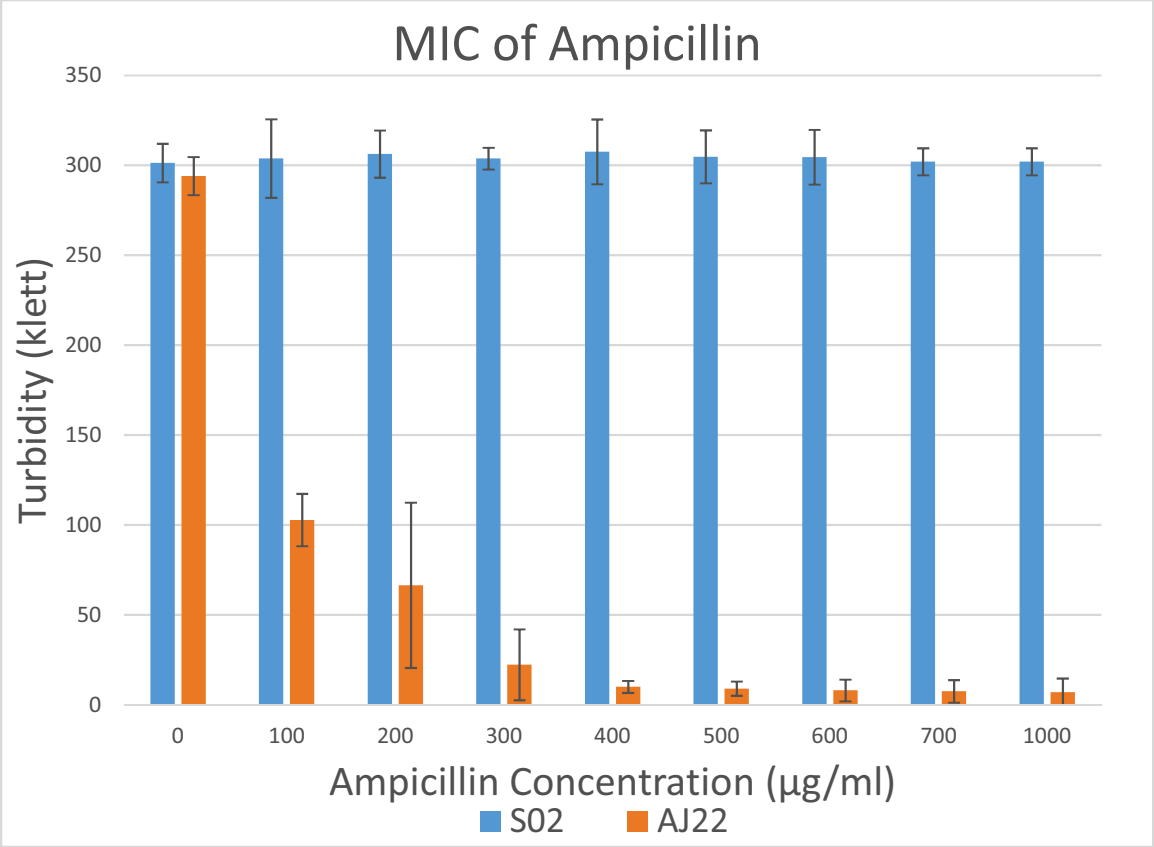


Figure 2: Minimum Inhibitory Concentration testing of ampicillin on *S. maltophilia* 02 and AJ22. The blue bars indicate *S. maltophilia* 02 and shows its resistance to ampicillin as the bars are consistently above 300 klett at all ampicillin concentrations. The orange bars indicate the AJ22 mutant and its sensitivity to ampicillin because as the ampicillin concentrations increase, the turbidity of AJ22 decreases.

4.3 Genomic DNA Digestions

Genomic DNA purifications were performed on an AJ22 culture. It was then digested with the restriction endonucleases, *Kpn*I and *Pvu*II which cut outside the transposon. The restriction endonucleases sheared the DNA into many pieces causing it to smear on the gel (Figure 3).

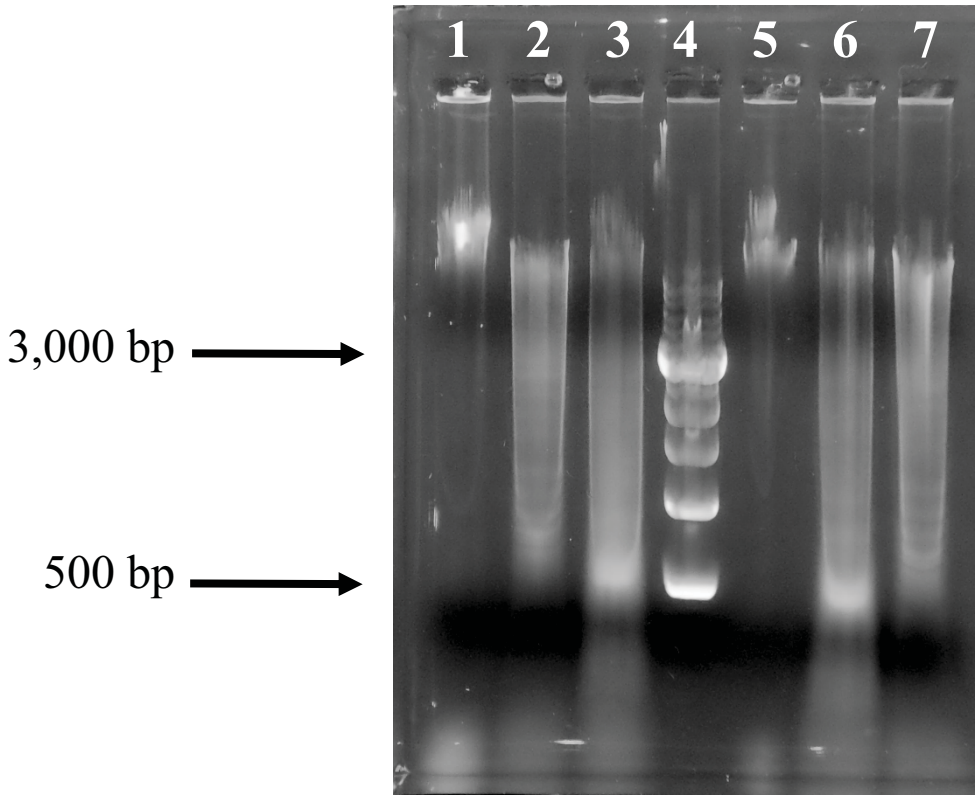


Figure 3: Gel Electrophoresis of genomic DNA Digestion. Lane 3 – 1kb ladder (with arrows indicating the 500 base pair and 3,000 base pair bands), Lanes 1 and 5 – undigested genomic DNA, Lanes 2 and 6 – DNA digested with *Kpn*I, Lanes 3 and 7 – DNA digested with *Pvu*II.

4.4 Gene Rescue

A T4 DNA ligation was then performed in order to circularize the digested DNA and create recombinant plasmids that contain the transposon, plus a flanking chromosomal region. The ligated DNA was then transformed by the calcium chloride method into ECD100D *pir*116 competent cells. Colonies grew on all of the kanamycin plates. This indicated a successful transformation since only colonies with the transposon with a flanking ampicillin resistance gene will grow.

Plasmid DNA Purification was performed to isolate and purify the plasmid DNA. The plasmid DNA was then digested with *Kpn*I and *Pvu*II again and gel electrophoresis was performed (Figure 4). All of the digested DNA was about 4,000 base pairs.

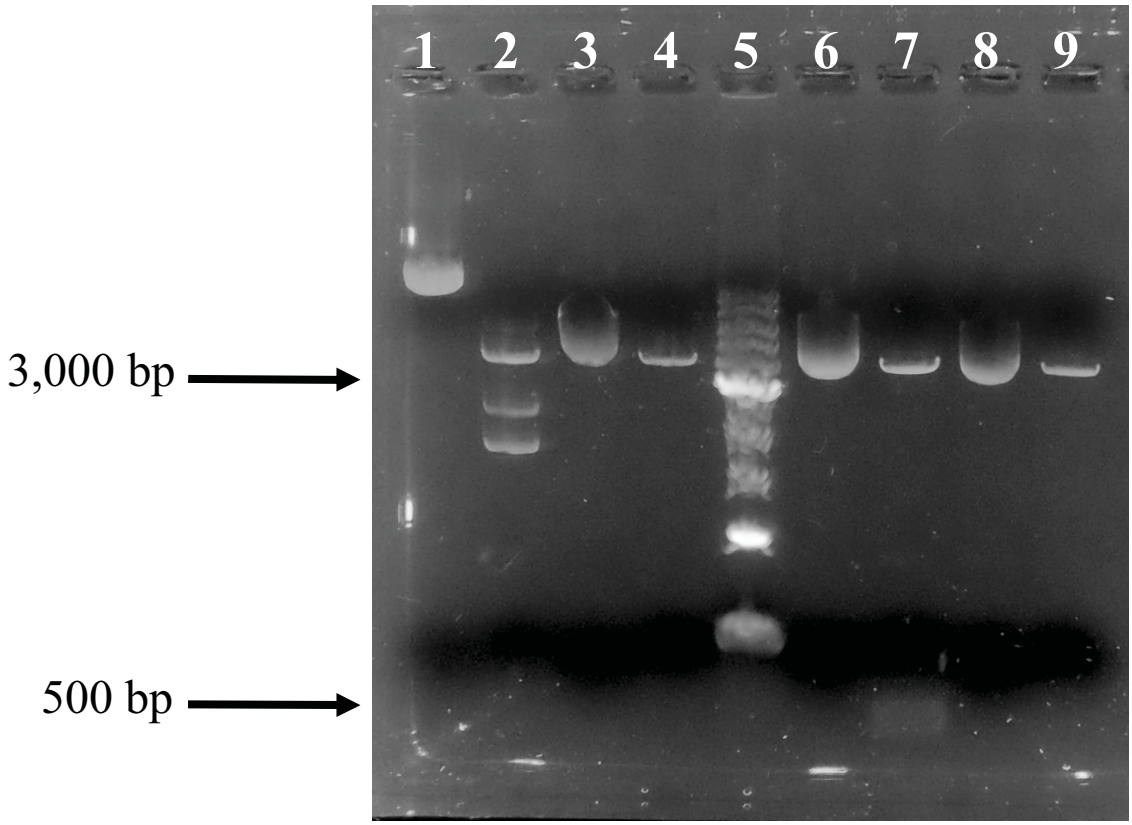


Figure 4: Gel Electrophoresis of purified plasmid DNA. Lane 5 – 1kb ladder (with arrows indicating the 500 base pair and 3,000 base pair bands), Lanes 1, 3, 6 and 8 – undigested DNA, Lanes 2 and 4 – DNA digested with *Kpn*I, Lanes 7 and 9 – DNA digested with *Pvu*II.

4.5 DNA Sequencing

DNA sequencing was performed using the primers KAN-2 FP-1 and R6KAN-2 RP-1 (Table 1). The resulting sequences were analyzed in BLAST. This showed that the sequence was part of the regulation gene *ampG* which encodes a transmembrane protein and is also involved in cell wall recycling (36).

Stenotrophomonas maltophilia strain KJ AmpN (ampN) and AmpG permease (ampG) genes, complete cds

Sequence ID: gb|GQ469998.1| **Length:** 2694 **Number of Matches:** 1

Range 1: 1134 to 2508 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
2071 bits(1121)	0.0	1292/1375(94%)	9/1375(0%)	Plus/Plus

```
Query  1     GTGACCGAGGCTGCCAAGCCGCGCCGGCCGTGGCAGCAGGTGGTTTCCAACCTGAGCCAG  60
Sbjct  1134  GTGACCGAGGCTTCCAAGCCGCGCCGACCGTGGCAGCAGGTGTTGTCCAACCTGAGCCAG  1193

Query  61    CGCAAGGTGCTGGCGATGCTGCTGCTCGGCTTCAGTTCCGGCCTGCCGATCTATCTGGTG  120
Sbjct  1194  CGCAAGGTGCTGGCGATGCTGCTGCTCGGCTTCAGTTCCGGCCTGCCGATCTATCTGGTG  1253

Query  121   GGCAACACGCTCGGCTTCTGGATGCGCAAGGAAGGCATCGAGCTGAGCACGATCGGTTTC  180
Sbjct  1254  GGCAACACGCTCGGCTTCTGGATGCGCAAGGAAGGCATCGAGCTGAGTACGATCGGCTTC  1313

Query  181   CTGTCATGGGTCGGGCTGGCCTACACCATGAAGTTCCTGTGGGCACCGATCGTCGACAAG  240
Sbjct  1314  CTGTCATGGGTCGGGCTGGCCTACACCATGAAGTTCCTGTGGGCACCGATCGTCGACAAG  1373

Query  241   ACCGACGTGCCTCTGTTCGGCCGCTTTGGCCGCCGCCGTGGCTGGATGCTGCTGTCGCAG  300
Sbjct  1374  ACCGACGTGCCTCTGTTCGGCCGCTTTGGCCGCCGCCGTGGCTGGATGCTGCTGTCGCAG  1433

Query  301   CTGGTTGTGGTGGTGGGCCTGGTCGGCATGGCGCTGGTCCAGCCGAAGGGGGGCCAGATC  360
Sbjct  1434  CTGGTCGTGATGGTGGGCCTGGTCGGCATGGCGCTGGTCCAGCCCAAGGGGGGCCAGATC  1493

Query  361   CAGTTCCTGGGCATCGCCTGGCAGCACATTGTCGTGTTCGGCGTGATGGCCGTGATCGTG  420
Sbjct  1494  CAGTTCCTGGGTATCGCCTGGCAGCACATCGTCGTGTTCGGCGTGATGGCCGTGATCGTA  1553

Query  421   GCGTTTGCTTCGGCCACGCAGGACATCGTCATCGATGCTTGGCGCATTGAAAGTGCTGAC  480
Sbjct  1554  GCGTTTGCTTCGGCCACCCAGGACATCGTCATCGATGCCTGGCGCATTGAAAGTGCTGAC  1613

Query  481   AACAGCGAGCAGCTTGGACTGCTGACCTCATCCTCTGCACTGGGCTACCGCACTGCATTG  540
Sbjct  1614  AACAGCGAGCAGCTCGGTCTGCTGACCTCATCATCCGCGCTGGGCTATCGCACCGCATTG  1673

Query  541   CTGGTTACCGATGCGCTGATCCTGATCATCGCGGCCCGTGTTGGCTGGCAGGTTTCCTAC  600
Sbjct  1674  CTGGTCACCGATGCGCTGATCCTGATCATCGCGGCCCGTGTCGGCTGGCAGGTCTCGTAC  1733

Query  601   GAGATCATGGCCGTGCTGATGGCGCTTGGCGTGGTGGCCGTGGTCATGGCCCGTGAACCC  660
Sbjct  1734  GAGATCATGGCGGTACTCATGGCGCTTGGCGTGGTGGCCGTGGTCATGGCCAGGGAACCC  1793

Query  661   GCGCGGGAAGTGGCTGCCGTGCAGGCACAGGCGACTTCCCTGTGGACGCCGCGCGGATTG  720
Sbjct  1794  GCCAGGGAGGTGGCTGCCGTGCAGGCGCAGGCGACCTCGCTGTGGACGCCGCGTGGCCTG  1853
```

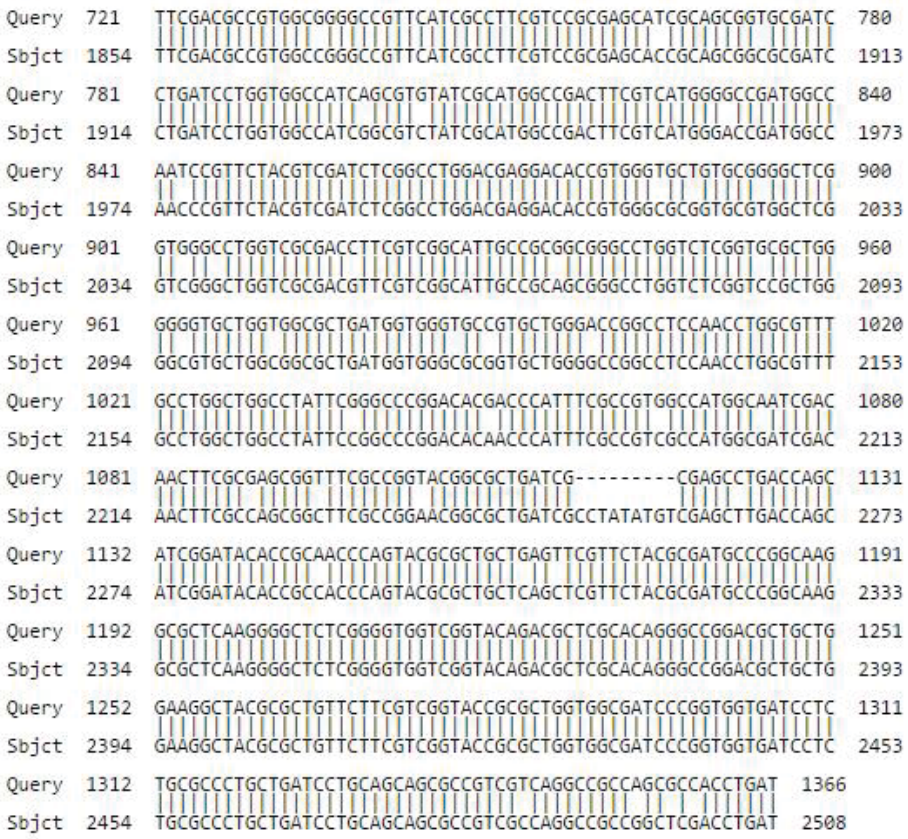
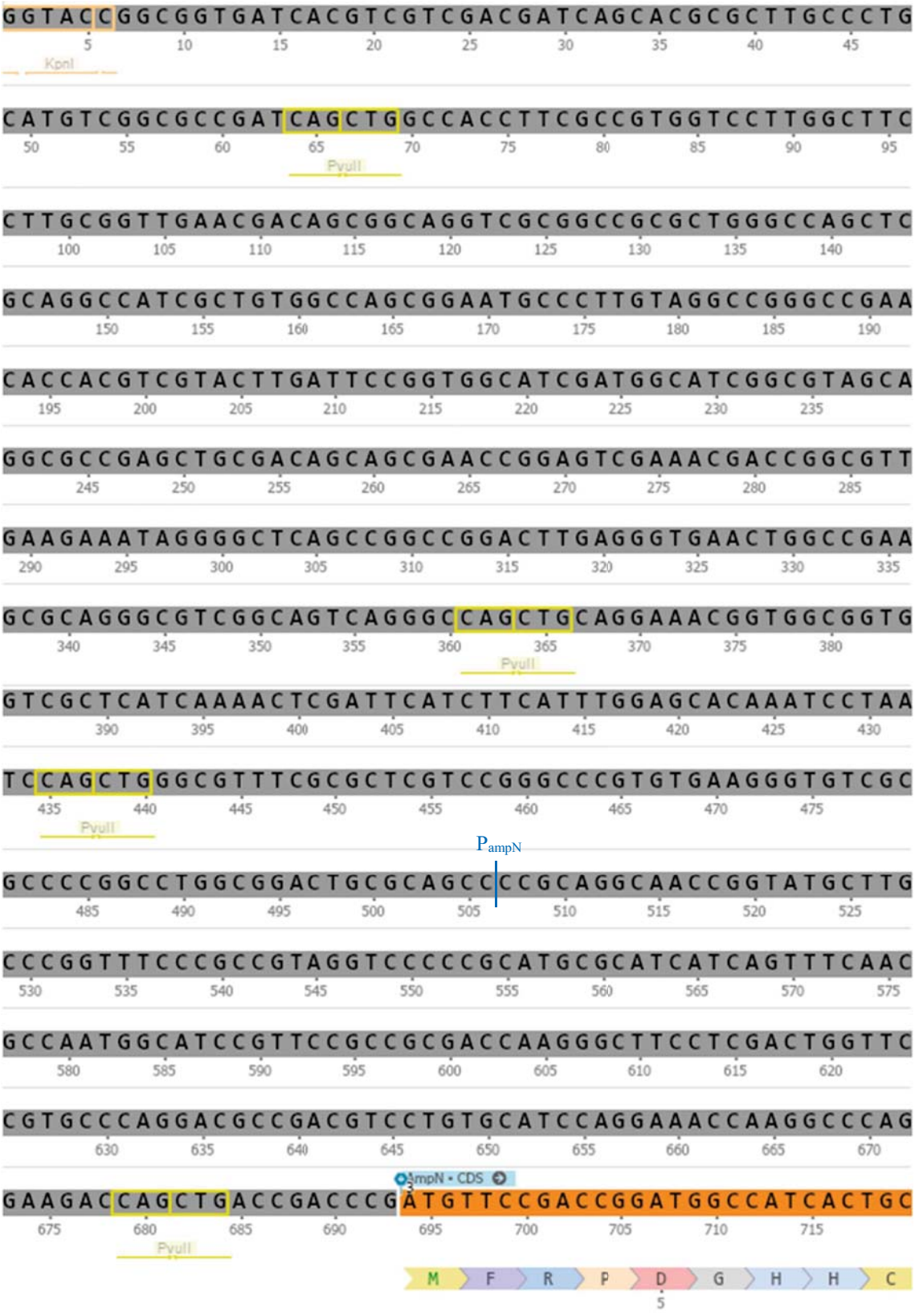


Figure 5: BLAST Analysis of DNA Sequence. This nucleotide BLAST (blastn) analysis matched the submitted sequence (query) to *Stenotrophomonas maltophilia* strain KJ AmpN (*ampN*) and AmpG permease (*ampG*) genes, complete cds (Sbjct) with a 94% identity match.

4.6 Sequence Analysis

The sequences were used to design new primers in order to sequence the rest of *ampG* (Table 1). The sequences were aligned using GeneStudio (37) and all the ambiguities were corrected. The consensus sequence was translated using ExPASy (32) and analyzed using protein BLAST. This matched the protein sequence to homologs. These homologs were then aligned with the *ampG* sequence using ClustalX (34) and then viewed in GeneDoc (Figure 8). The *ampN* (Figure 6) and *ampG* (Figure 7) amino acid and protein sequences were viewed in Genome Compiler.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KpnI
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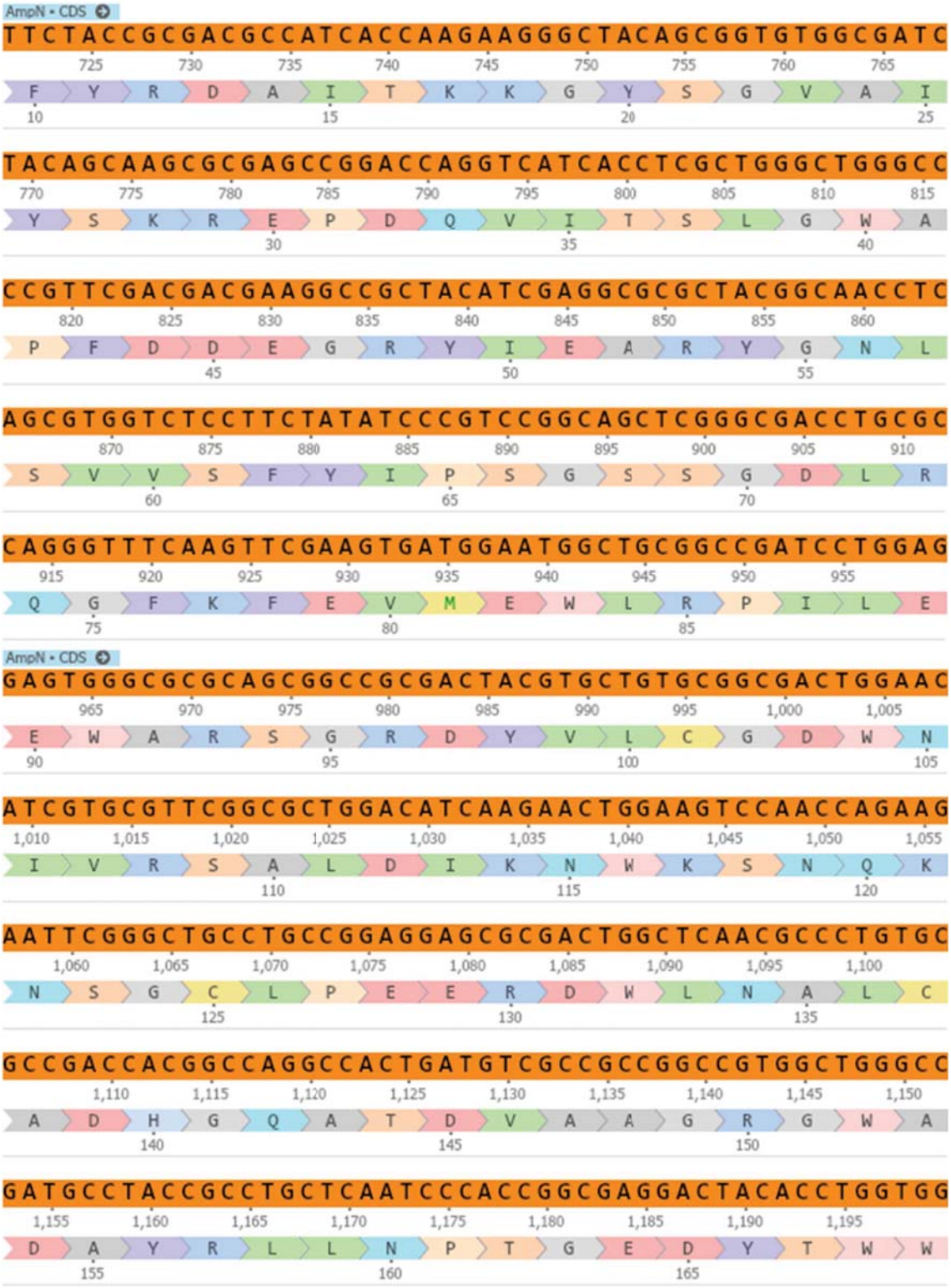
50 55 60 65 70 75 80 85 90 95
PvuII
CTTGCGGTTGAACGACAGCGGCAGGTCGCGGCCGCGCTGGGCCAGCTC
100 105 110 115 120 125 130 135 140
GCAGGCCATCGCTGTGGCCAGCGGAATGCCCTTGTAGGCCGGGCCGAA
150 155 160 165 170 175 180 185 190
CACCACGTCGTACTTGATTCCGGTGGCATCGATGGCATCGGCGTAGCA
195 200 205 210 215 220 225 230 235
GGCGCCGAGCTGCGACAGCAGCGAACCGGAGTCGAAACGACCGGCGTT
245 250 255 260 265 270 275 280 285
GAAGAAATAGGGGCTCAGCCGGCCGGACTTGAGGGTGAACTGGCCGAA
290 295 300 305 310 315 320 325 330 335
GCGCAGGGCGTCGGCAGTCAGGGCCAGCTGCAGGAAACGGTGGCGGTG
340 345 350 355 360 365 370 375 380
PvuII
GTCGCTCATCAAAACTCGATTCATCTTCATTTGGAGCACAAATCCTAA
390 395 400 405 410 415 420 425 430
TCCAGCTGGGCGTTTCGCGCTCGTCCGGGCCCGTGTGAAGGGTGTCGC
435 440 445 450 455 460 465 470 475
PvuII
PampN
GCCCCGGCCTGGCGGACTGCGCAGCCCCGCAGGCAACCGGTATGCTTG
485 490 495 500 505 510 515 520 525
CCCGGTTTCCCGCCGTAGGTCCCCCGCATGCGCATCATCAGTTTCAAC
530 535 540 545 550 555 560 565 570 575
GCCAATGGCATCCGTTCCGCCGCGACCAAGGGCTTCCTCGACTGGTTC
580 585 590 595 600 605 610 615 620
CGTGCCCAGGACGCCGACGTCCTGTGCATCCAGGAAACCAAGGCCCAG
630 635 640 645 650 655 660 665 670
ampN - CDS
GAAGACCAGCTGACCGACCCGATGTTCCGACCGGATGGCCATCACTGC
675 680 685 690 695 700 705 710 715
PvuII
M F R P D G H H C
5


AmpN • CDS
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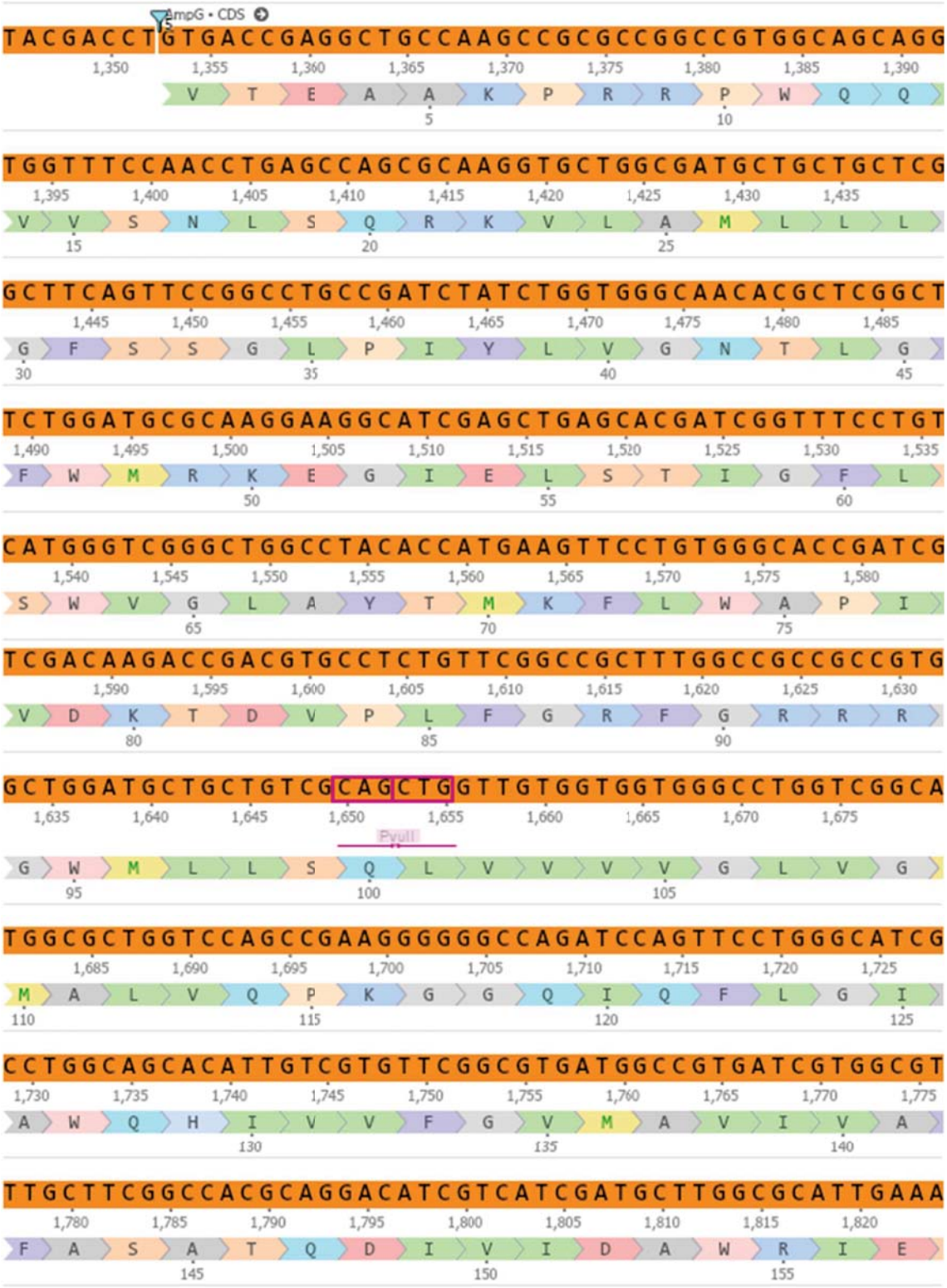
725 730 735 740 745 750 755 760 765
F Y R D A I T K K G Y S G V A I
10 15 20 25
TACAGCAAGCGCGAGCCGGACCAGGTCATCACCTCGCTGGGCTGGGCC
770 775 780 785 790 795 800 805 810 815
Y S K R E P D Q V I T S L G W A
30 35 40
CCGTTCGACGACGAAGGCCGCTACATCGAGGCGCGCTACGGCAACCTC
820 825 830 835 840 845 850 855 860
P F D D E G R Y I E A R Y G N L
45 50 55
AGCGTGGTCTCCTTCTATATCCCGTCCGGCAGCTCGGGCGACCTGCGC
870 875 880 885 890 895 900 905 910
S V V S F Y I P S G S S G D L R
60 65 70
CAGGGTTTCAAGTTCGAAGTGATGGAATGGCTGCGGCCGATCCTGGAG
915 920 925 930 935 940 945 950 955
Q G F K F E V M E W L R P I L E
75 80 85
AmpN • CDS
GAGTGGGCGCGCAGCGGCCGCGACTACGTGCTGTGCGGCGACTGGAAC
965 970 975 980 985 990 995 1,000 1,005
E W A R S G R D Y V L C G D W N
90 95 100 105
ATCGTGCGTTCGGCGCTGGACATCAAGAACTGGAAGTCCAACCAGAAG
1,010 1,015 1,020 1,025 1,030 1,035 1,040 1,045 1,050 1,055
I V R S A L D I K N W K S N Q K
110 115 120
AATTCGGGCTGCCTGCCGGAGGAGCGCGACTGGCTCAACGCCCTGTGC
1,060 1,065 1,070 1,075 1,080 1,085 1,090 1,095 1,100
N S G C L P E E R D W L N A L C
125 130 135
GCCGACCACGGCCAGGCCACTGATGTCGCCGCCGGCCGTGGCTGGGCC
1,110 1,115 1,120 1,125 1,130 1,135 1,140 1,145 1,150
A D H G Q A T D V A A G R G W A
140 145 150
GATGCCTACCGCCTGCTCAATCCCACCGGCGAGGACTACACCTGGTGG
1,155 1,160 1,165 1,170 1,175 1,180 1,185 1,190 1,195
D A Y R L L N P T G E D Y T W W
155 160 165

Figure 6: *ampN* sequence in Genome Compiler. The promoter, P_{ampN}, starts at 507 bp and is 187 bp long. *ampN* starts at 694 bp with the amino acid sequence shown in orange and the protein sequence shown below it. The restriction enzymes *Pvu*II and *Kpn*I are also labeled.


ampG • CDS
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V T E A A K P R R P W Q Q
5 10
TGGTTTCCAACCTGAGCCAGCGCAAGGTGCTGGCGATGCTGCTGCTCG
1,395 1,400 1,405 1,410 1,415 1,420 1,425 1,430 1,435
V V S N L S Q R K V L A M L L L
15 20 25
GCTTCAGTTCCGGCCTGCCGATCTATCTGGTGGGCAACACGCTCGGCT
1,445 1,450 1,455 1,460 1,465 1,470 1,475 1,480 1,485
G F S S G L P I Y L V G N T L G
30 35 40 45
TCTGGATGCGCAAGGAAGGCATCGAGCTGAGCACGATCGGTTTCCTGT
1,490 1,495 1,500 1,505 1,510 1,515 1,520 1,525 1,530 1,535
F W M R K E G I E L S T I G F L
50 55 60
CATGGGTCGGGCTGGCCTACACCATGAAGTTCCTGTGGGCACCGATCG
1,540 1,545 1,550 1,555 1,560 1,565 1,570 1,575 1,580
S W V G L A Y T M K F L W A P I
65 70 75
TCGACAAGACCGACGTGCCTCTGTTCGGCCGCTTTGGCCGCCGCCGTG
1,590 1,595 1,600 1,605 1,610 1,615 1,620 1,625 1,630
V D K T D V P L F G R F G R R R
80 85 90
GCTGGATGCTGCTGTCGCAGCTGGTTGTGGTGGTGGGCCTGGTCGGCA
1,635 1,640 1,645 1,650 1,655 1,660 1,665 1,670 1,675
PvuII
G W M L L S Q L V V V V G L V G
95 100 105
TGGCGCTGGTCCAGCCGAAGGGGGGCCAGATCCAGTTCCTGGGCATCG
1,685 1,690 1,695 1,700 1,705 1,710 1,715 1,720 1,725
M A L V Q P K G G Q I Q F L G I
110 115 120 125
CCTGGCAGCACATTGTCGTGTTCGGCGTGATGGCCGTGATCGTGGCGT
1,730 1,735 1,740 1,745 1,750 1,755 1,760 1,765 1,770 1,775
A W Q H I V V F G V M A V I V A
130 135 140
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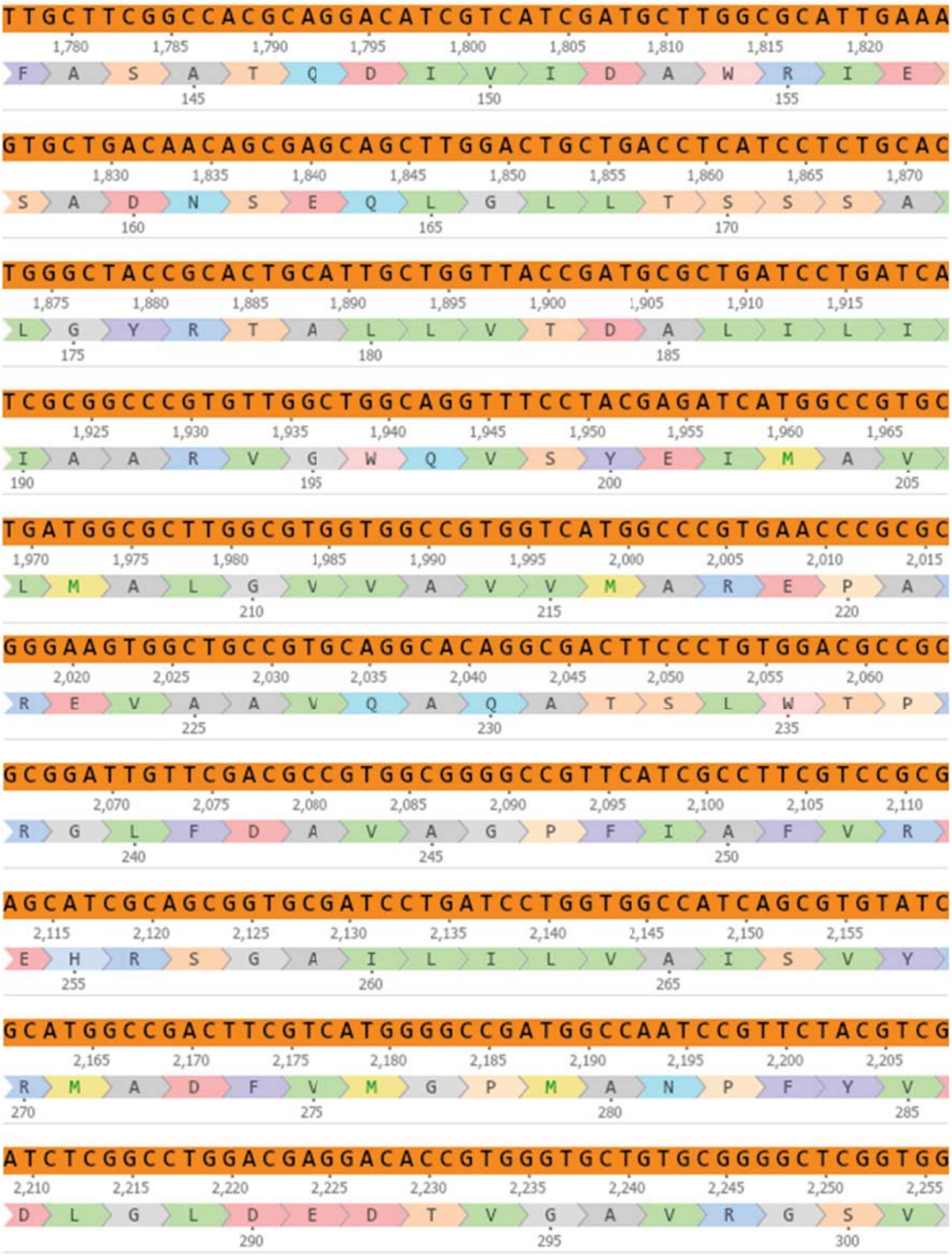
145 150 155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1,780 1,785 1,790 1,795 1,800 1,805 1,810 1,815 1,820
F A S A T Q D I V I D A W R I E
145 150 155
GTGCTGACAACAGCGAGCAGCTTGGACTGCTGACCTCATCCTCTGCAC
1,830 1,835 1,840 1,845 1,850 1,855 1,860 1,865 1,870
S A D N S E Q L G L L T S S S A
160 165 170
TGGGCTACCGCACTGCATTGCTGGTTACCGATGCGCTGATCCTGATCA
1,875 1,880 1,885 1,890 1,895 1,900 1,905 1,910 1,915
L G Y R T A L L V T D A L I L I
175 180 185
TCGCGGCCCGTGTTGGCTGGCAGGTTTCCTACGAGATCATGGCCGTGC
1,925 1,930 1,935 1,940 1,945 1,950 1,955 1,960 1,965
I A A R V G W Q V S Y E I M A V
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L M A L G V V A V V M A R E P A
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GGGAAGTGGCTGCCGTGCAGGCACAGGCGACTTCCCTGTGGACGCCGC
2,020 2,025 2,030 2,035 2,040 2,045 2,050 2,055 2,060
R E V A A V Q A Q A T S L W T P
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2,070 2,075 2,080 2,085 2,090 2,095 2,100 2,105 2,110
R G L F D A V A G P F I A F V R
240 245 250
AGCATCGCAGCGGTGCGATCCTGATCCTGGTGGCCATCAGCGTGTATC
2,115 2,120 2,125 2,130 2,135 2,140 2,145 2,150 2,155
E H R S G A I L I L V A I S V Y
255 260 265
GCATGGCCGACTTCGTCATGGGGCCGATGGCCAATCCGTTCTACGTCG
2,165 2,170 2,175 2,180 2,185 2,190 2,195 2,200 2,205
R M A D F V M G P M A N P F Y V
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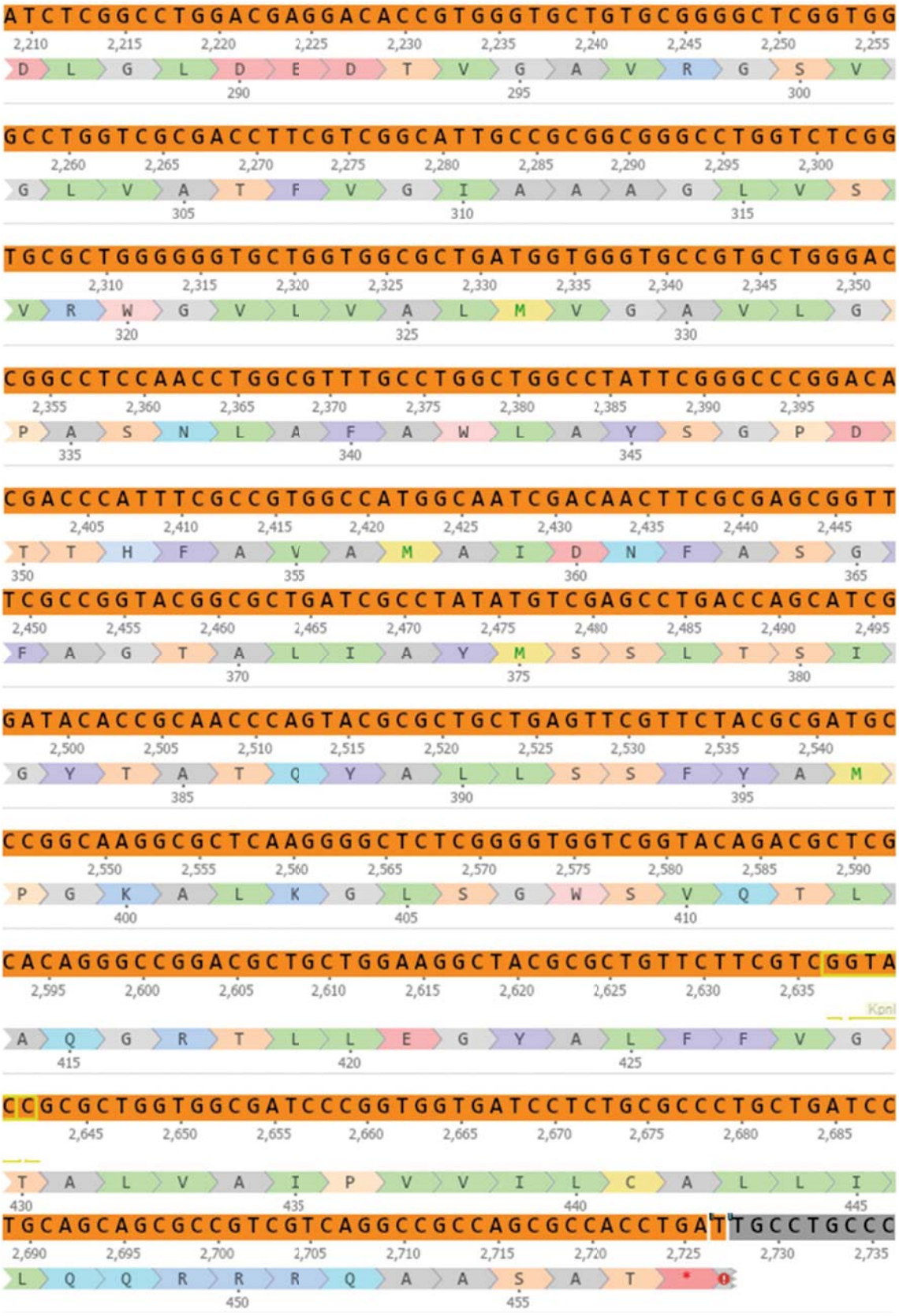
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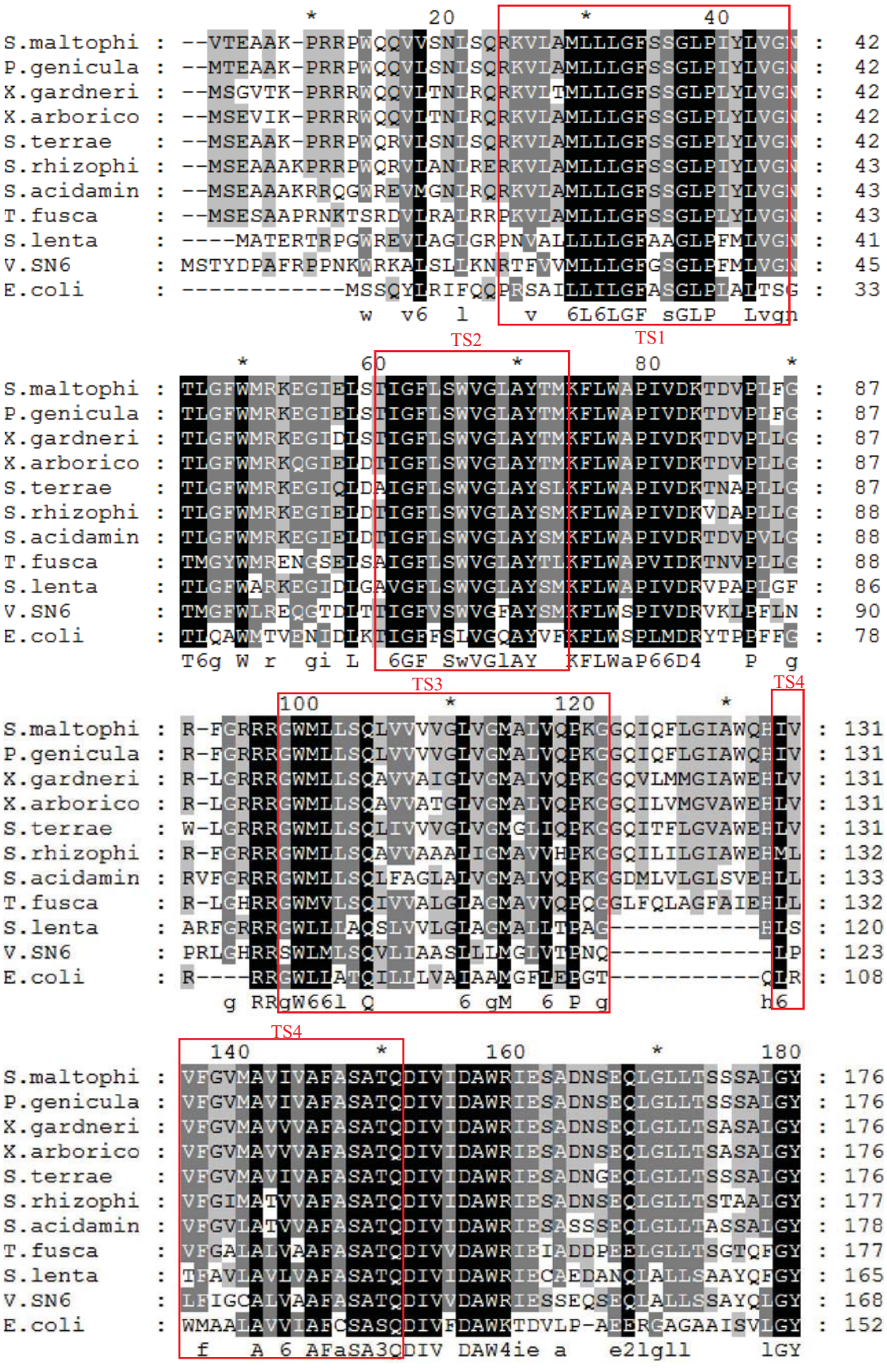
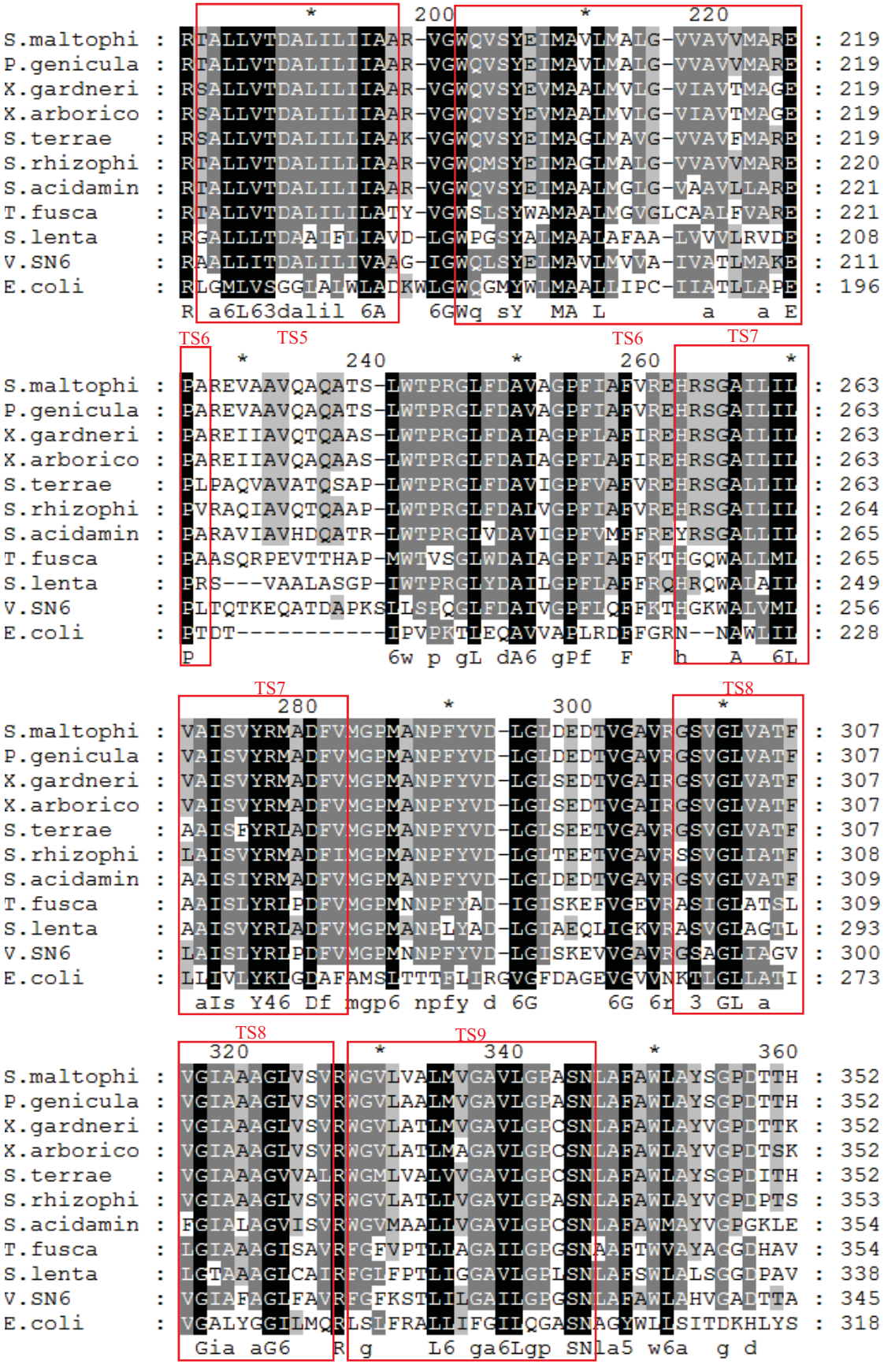
D L G L D E D T V G A V R G S V
290 295 300
GCCTGGTCGCGACCTTCGTCGGCATTGCCGCGGCGGGGCCTGGTCTCGG
2,260 2,265 2,270 2,275 2,280 2,285 2,290 2,295 2,300
G L V A T F V G I A A A G L V S
305 310 315
TGCGCTGGGGGGTGCTGGTGGCGCTGATGGTGGGTGCCGTGCTGGGAC
2,310 2,315 2,320 2,325 2,330 2,335 2,340 2,345 2,350
V R W G V L V A L M V G A V L G
320 325 330
CGGCCTCCAACCTGGCGTTTGCCTGGCTGGCCTATTCGGGCCCGGACA
2,355 2,360 2,365 2,370 2,375 2,380 2,385 2,390 2,395
P A S N L A F A W L A Y S G P D
335 340 345
CGACCCATTTCGCCGTGGCCATGGCAATCGACAACTTCGCGAGCGGTT
2,405 2,410 2,415 2,420 2,425 2,430 2,435 2,440 2,445
T T H F A V A M A I D N F A S G
350 355 360 365
TCGCCGGTACGGCGCTGATCGCCTATATGTCGAGCCTGACCAGCATCG
2,450 2,455 2,460 2,465 2,470 2,475 2,480 2,485 2,490 2,495
F A G T A L I A Y M S S L T S I
370 375 380
GATACACCGCAACCCAGTACGCGCTGCTGAGTTCGTTCTACGCGATGC
2,500 2,505 2,510 2,515 2,520 2,525 2,530 2,535 2,540
G Y T A T Q Y A L L S S F Y A M
385 390 395
CCGGCAAGGCGCTCAAGGGGCTCTCGGGGTGGTCGGTACAGACGCTCG
2,550 2,555 2,560 2,565 2,570 2,575 2,580 2,585 2,590
P G K A L K G L S G W S V Q T L
400 405 410
CACAGGGCCGGACGCTGCTGGAAGGCTACGCGCTGTTCTTCGTCGGTA
2,595 2,600 2,605 2,610 2,615 2,620 2,625 2,630 2,635
KpnI
A Q G R T L L E G Y A L F F V G
415 420 425
CCGCGCTGGTGGCGATCCCGGTGGTGATCCTCTGCGCCCTGCTGATCC
2,645 2,650 2,655 2,660 2,665 2,670 2,675 2,680 2,685
T A L V A I P V V I L C A L L I
430 435 440 445
TGCAGCAGCGCCGTCGTCAGGCCGCCAGCGCCACCTGATTGCCTGCCC
2,690 2,695 2,700 2,705 2,710 2,715 2,720 2,725 2,730 2,735
L Q Q R R R Q A A S A T * Q
450 455

Figure 7: *ampG* sequence in Genome Compiler. *ampG* starts at 1,353 bp with the amino acid sequence shown in orange and the protein sequence shown below it. The beginning of *ampG* overlaps with 4 bp from the end of *ampN*, GTGA. The restriction enzymes *Pvu*II and *Kpn*I are also labeled.


TS2
TS1
TS3
TS4
TS4


TS6
TS5
TS6
TS7
TS7
TS8
TS8
TS9

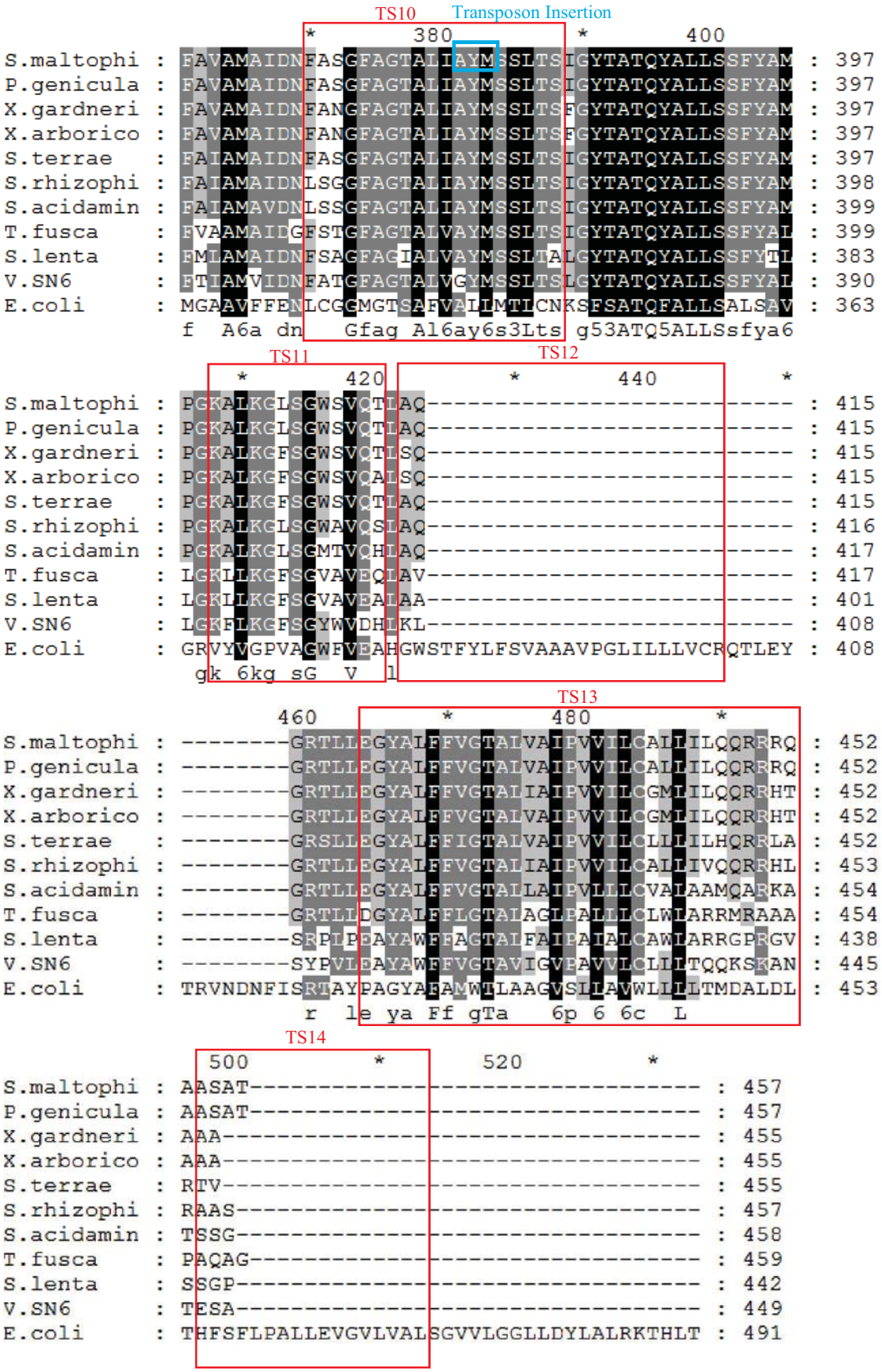

TS10
Transposon Insertion
TS11
TS12
TS13
TS14
S.maltophi
P.genicula
X.gardneri
X.arborico
S.terrae
S.rhizophi
S.acidamin
T.fusca
S.lenta
V.SN6
E.coli

Figure 8: Multiple Sequence Alignment. The alignment of the *S. maltophilia ampG* protein sequence and its homologs using GeneDoc. *E. coli ampG* was used as a reference sequence to determine where the transmembrane sequences (TS) were located. These are shown in the red boxes labeled TS1-14. The blue box indicated where the transposon was inserted.

4.7 Phylogenetic Analysis

A phylogenetic tree was constructed to compare the evolutionary relationship between the *ampG* gene in *S. maltophilia* and its homologs (Figure 7). This shows that *S. maltophilia* is more closely related to *Pseudomonas geniculata* than the other *Stenotrophomonas* strains.

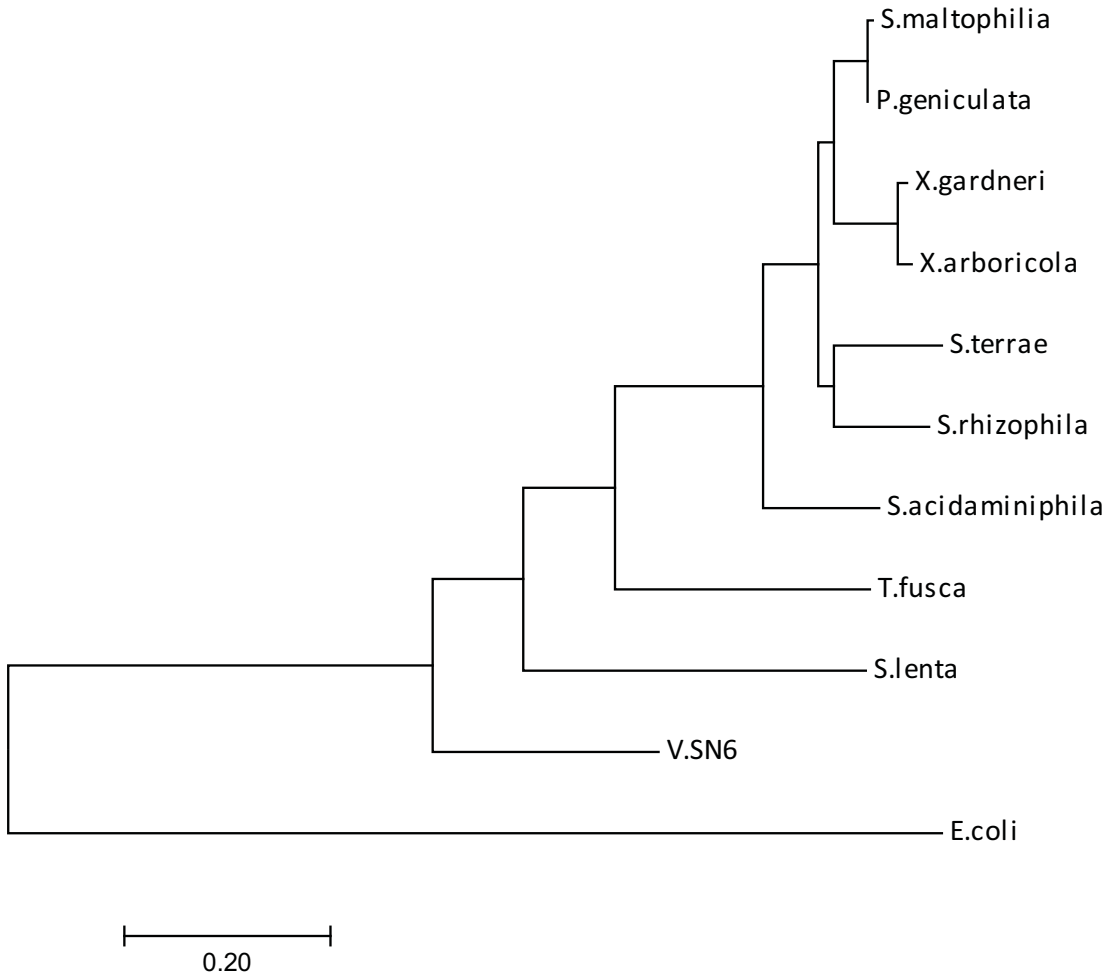


Figure 9: Phylogenetic Analysis. Phylogenetic tree showing the relationship between *S. maltophilia* and its homologs.

4.8 Southern Blotting

Southern Blotting was performed to show that the transposon only inserted itself into *ampG*. *S. maltophilia* and AJ22 genomic DNA was digested with *Kpn*I and *Pvu*II and then gel electrophoresis was performed (Figure 10). This was blotted onto a positively charged Biodyne® B Pre-Cut Modified Nylon Membrane. The probe hybridized to the positive control in Lane 2 of Figure 11. There were no signals in Lanes 3 and 5 which were the negative controls. These contained digested S02 genomic DNA and lacked the EZ-Tn*5* transposon. Lanes 4 and 6 each contained 1 band therefore showing that the transposon was only inserted into one site.

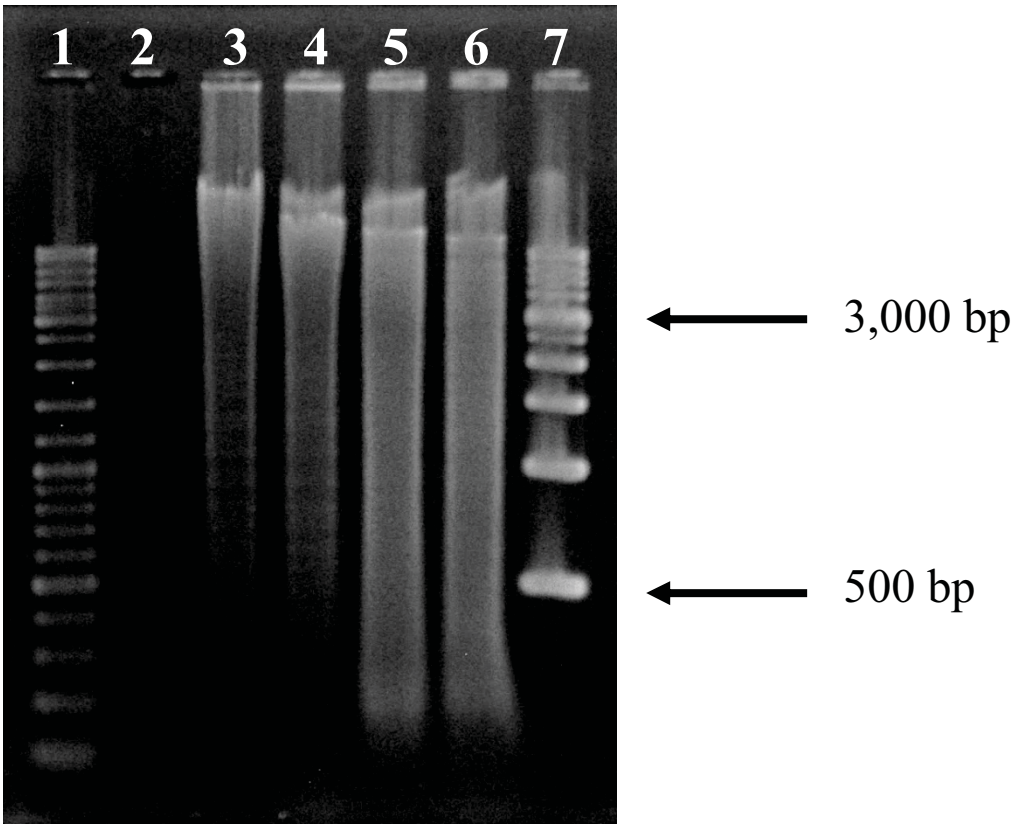


Figure 10: Gel Electrophoresis of Southern Blotting. Lane 1 - biotin labeled ladder, Lane 2 – Tn*5*, Lane 3 – S02 *Kpn*I digestion, Lane 4 – AJ22 *Kpn*I digestion, Lane 5 – S02 *Pvu*II digestion, Lane 6 – AJ22 *Pvu*II digestion, Lane 7 – 1 kb ladder.

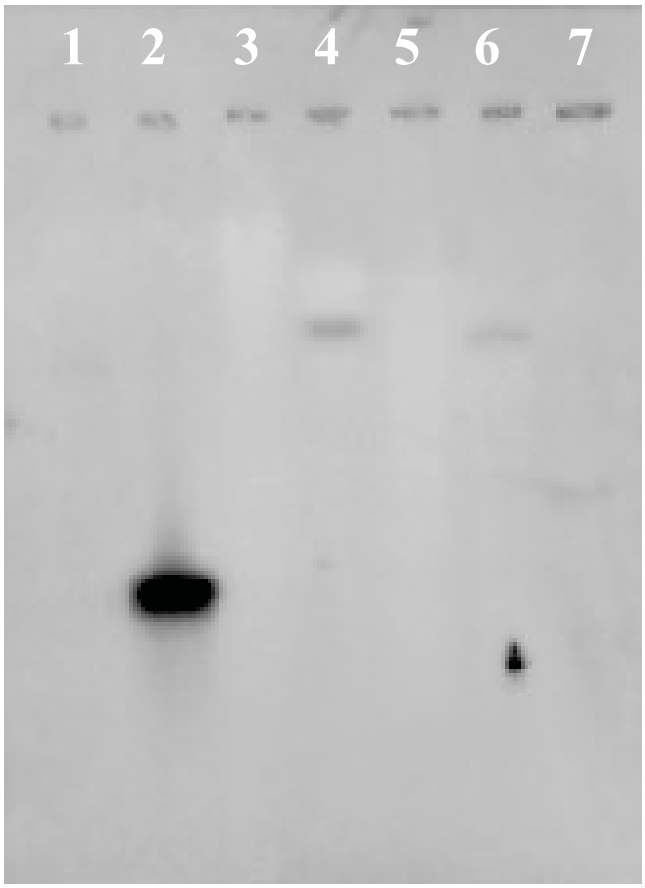


Figure 11: Southern Blot Detection. Lane 1 - biotin labeled ladder, Lane 2 – Tn*5*, Lane 3 – S02 *Kpn*I digestion, Lane 4 – AJ22 *Kpn*I digestion, Lane 5 – S02 *Pvu*II digestion, Lane 6 – AJ22 *Pvu*II digestion, Lane 7 – 1 kb ladder.

Chapter V: Discussion

As hypothesized the transposon interrupted a putative *ampG* gene, which encodes a protein involved in regulating β-lactamase activity. Since *Stenotrophomonas maltophilia* OR02 probably contains two ampicillin resistance genes, L1 and L2, it was unlikely that the transposon interrupted both genes. *ampN* forms an operon with *ampG.* This sequence is frequently annotated as DNA-(apurinic or apyrimidinic site) lyase in the BLAST database. However, one study has determined that this may be *ampN* (28).

5.1 *ampG*

In *Pseudomonas aeruginosa* PAO1, *ampG* also has a role in murein recycling and transports N-acetylglucosamine anhydrous N-acetylmuramyl peptides across the inner membrane. When *ampG* is mutated there is an effect on the resistance to β-lactam antibiotics. *P. aeruginosa*'*s ampG* is different from others because the genome has two *ampG* orthologs that were named *ampP* and *ampG*. β-galactosidase transcriptional fusions were used to determine that *ampG* operon expression is β-lactam and *ampR*-dependent and independent expression of *ampG* relies on *ampP*. *AmpP* expression is autoregulated and also regulates *ampG*. Both of their topologies are consistent with transportation functions. *AmpG* in *P. aeruginosa* contains of 14 transmembrane helices and *ampP* has 10 (38).

AmpG is required for β-lactamase induction. AmpG is also a permease that is necessary during the recycling of murein tripeptide and the uptake of anhydromuropeptides. It is the permease for the disaccharide, N-acetylglucosaminyl-β-1,4-anhydro-N-acetylmuramic acid (GlcNAc-anhMurNAc), whose presence is the main

requirement for uptake to occur. If the muropeptide lacks either GlcNAc or anhMurNAc, transportation will not occur. This was determined because anhydro-N-acetylmuramyl-L-alanyl-γ-D-glutamyl-meso-diaminopimelic acid (anhMurNAc-tripeptide) does not accumulate in *ampG* cells. The breakdown of the murein sacculus produces GlcNAc-anhMurNAc-peptides. AmpG permease is sensitive to carbonylcyanide *m*-chlorophenylhydrazone, which prevents the uptake of GlcNAc- anhMurNAc and GlcNAc-anhMurNAc-peptides. It can therefore be determined that it is a single-component permease and transport relies on the proton motive force (39).

In a proposed model of *E. coli* AmpG, there are 10 transmembrane hydrophobic segments (TS1, TS2, TS3, TS4, TS7, TS8, TS9, TS10, TS13 and TS14) (Figure 6). These delimit five periplasmic hydrophilic domains and six cytoplasmic hydrophilic domains including the N- and C-terminal ends. There are also two large cytoplasmic loops which contain four transmembrane segments that are located in the cytoplasm (TS5, TS6, TS11 and TS12) (Figure 6) and could be involved in a scissors-type mechanism. *E. coli* and most other gram-negative bacteria only lose about 5-8% of their peptidoglycan per generation in comparison to the gram-positive *Bacillus subtilis* which can lose up to 30%. This difference can be attributed to the presence of efficient protein machinery which recycles the peptidoglycan. The main product that is recycled by uptake with AmpG is N-acetylglucosamine-1,6-anhydro-N-acetylmuramic acid-tetrapeptide (GlucNAc-anhydro-MurNAc-tetrapeptide or anhydromuropeptide) (40). The anhydromuropeptide gets degraded in the cytoplasm and releases GlcNAc, aMurNAc, D-alanine, and the murein tripeptide and then the tripeptide can reenter the peptidoglycan biosynthesis pathway

(41). The *E. coli ampG* gene encodes a 491 amino acid protein and has a 53 kDa molecular mass (40).

5.2 *ampG* in *S. maltophilia*

After the AmpN/AmpG permease system transports degraded peptidoglycan fragments into the cytosol, they are processed into activator ligands for β-lactamase induction with assistance from AmpR (42). In *S. maltophilia, ampG* and *ampN*, an upstream reading frame (ORF), form an operon which is necessary for L1 and L2 β-lactamase induction. During *in silico* analysis of the *ampG* locus, S. *maltophilia* K279a was found to contain *ampG, ampN* and a hypothetical protein. A 663 bp ORF encoding the AmpN protein and a 1374 bp ORF encoding the AmpG protein were identified. There was insignificant signal peptide prediction so it was determined that AmpN is located in the cytoplasm. Because of the high percentage of similarity between other sequences, *ampN* and *ampG* were suggested to be highly conserved alleles in *S. maltophilia*. However, when compared to homologues the AmpG protein from strain KJ had less than a 58% match (28).

AmpG was predicted to contain 12 transmembrane segments (TM) with the N and C termini in the cytoplasm. The *ampN* and *ampG* genes overlap by four nucleotides and there is an intergenic region of 77 bp between *ampG* and a hypothetical protein gene. The *ampN-ampG* operon in *S. maltophilia* resembles the *yaiG-ampG* operon in *E. coli*. However, the *ampN* and *ampG* transcripts are almost equivalently expressed in the operon while in the *yaiG-ampG* operon, the expression of *ampG* seems to be lower. *ampG* does not have its own promoter. The promoter P_{ampN} codrives *ampG* and *ampN*

and is found in a 187 bp DNA fragment located upstream of *ampG.* AmpG is essential for β-lactamase induction because it most likely transports the precursor of a possible induction ligand. If the *ampN* gene is disrupted it exerts a polar effect on the expression of the *ampG* gene. The transcription of *ampG* could be impaired by the presence of an upstream Δ*ampN* allele. Both AmpN and AmpG are essential for β-lactamase expression. Similar to *E. coli*, the *ampN-ampG* operon displays a non-dosage effect on β-lactamase activity (28).

This data was consistent with *S. maltophilia* OR02. *ampN* is 801 base pairs and *ampG* is 1374 base pairs. The *ampN* and *ampG* genes also overlap by four nucleotides. Twelve of the fourteen transmembrane domains found in *E.*coli were also in *S. maltophilia*. TS12 and TS14 are not present so these may not be important for *ampG* in this bacterium.

Future Work

S. maltophilia AJ22 can be cloned to see if the ampicillin resistance can be restored. The L1 and L2 β-lactamase genes will also be cloned and sequenced by aligning the known L1 and L2 *S. maltophilia* sequences and designing PCR primers. qPCR will then be used to study the expression of L1 and L2.

Chapter VI: References

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2. **Brooks SC**. 2001. Waste Characteristics of the Former S-3 Ponds and Outline of Uranium Chemistry Relevant to NABIR Field Research Center Studies. Ornl/Tm-2001/27 21.

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Appendix 1

Species	**Accession**
Escherichia coli	WP_000098429 (40)
Pseudomonas geniculate	WP_057503977
Silanimonas lenta	WP_028770725.1
Stenotrophomonas acidaminiphila	ALJ27019.1
Stenotrophomonas maltophilia	Not Submitted
Stenotrophomonas rhizophila	AOA70942.1
Stenotrophomonas terrae	WP_057629665
Thermomonas fusca	WP_028839465.1
Vitreocilla sp.SN6	WP_058355686.1
Xanthomonas arboricola	WP_047129962.1
Xanthomonas gardneri	WP_043907423.1