CONSTRUCTION OF CELL-BASED ANTIBIOTIC RESISTANCE ARRAYS

CONSTRUCTION OF CELL-BASED ANTIBIOTIC RESISTANCE ARRAYS

By

ARLENE D. SUTHERLAND, H.B.Sc.

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2008) McMaster University
(Biochemistry) Hamilton, Ontario

TITLE: Construction of Cell-based Antibiotic Resistance Arrays

AUTHOR: Arlene D. Sutherland, H.B.Sc. (McMaster University)

SUPERVISOR: Professor G.D. Wright

NUMBER OF PAGES: x, 81

Abstract

As the problem of resistance increases in the current health care system, new solutions to this problem are not emerging at a similar rate. The ability to discover novel antibiotics, and modify existing antibiotics, is competing with highly evolving resistance profiles. An alternate solution to this problem may be to search for inhibitors of these resistance mechanisms and pairing them with current antibiotics. Proof of this hypothesis lies in the great success of β-lactamase inhibitors already in the clinic. Inhibitors may be created using synthetic methods, however searching for inhibitors found in the natural environment may lead to a greater success. For example, bacteria in their natural setting must cope with constant exposure to antibiotics secreted by both themselves and by other species. As well, bacteria must be able to handle encounters with other species that are resistant to their own defense mechanisms. With this in consideration, it is possible that these bacteria have already established an ability to challenge resistance encountered in their own environment, such as through the secretion of compounds that inhibit these mechanisms. Screening of such inhibitors can be done against purified resistance elements or via cell-based screens with resistant bacteria. The focus of this research was to develop expression systems which contain inducible antibiotic resistance genes to be used for whole-cell screening for inhibitors of antibiotic resistance. The expression systems studied were pSWEET, for use in the Gram positive bacterium *Bacillus subtilis,* and pETcoco, for use in the Gram negative bacterium *Escherichia coli.* It was found that the pSWEET expression system integrated into the *B. subtilis* chromosome at unspecified locations and was not an ideal system for the proposed screen. pETcoco holds promise as

a suitable expression system but at this point in time it requires further examination to ensure plasmid stability and reproducibility of results. Therefore further examination of these two systems is needed if they are to be used in a screen for inhibitors, and a search for substitute systems must be undertaken.

Acknowledgements

I would like to thank my mom and my sister for always being there for me and encouraging me to believe in myself. Your love and support did not go unnoticed and it helped me to find strength in times when I needed it most. I would also like to thank Josh for always being there for me during the busy days and stressful nights, and for always finding the positives in all situations. I don't know how I could have done it without you. I also thank God for always being by my side and willing me to persevere.

I would like to sincerely thank my supervisor, Dr. Gerry Wright, for the opportunity to be part of such a wonderful lab. Thank you for your patience, understanding, and for always believing in me. Your enthusiasm for science was truly inspiring. Additionally I would like to thank all of the members who were part of my committee, Dr. Lori Burrows, Dr. Christian Baron, Dr. Justin Nodwell, Dr. Murray Junop, and Dr. Joaquin Ortega, for their guidance, input and advice throughout my project.

Thank you to all of my close friends for understanding when I was always too busy to go out, or to stressed out to have any fun. You always cheered me up and shed light on some of my darkest moments. I would especially like to thank my best friend Katie for her encouragement, understanding, and her undying faith in me.

Finally, I would like to thank the members of the Wright lab. Thank you all of your help, advice, and for all the good times, you've made it an unforgettable two years. I would also like to give special thanks to Trish for all of your help and for being an incredible bay-mate, and to Tushar for enduring my endless strings of questions.

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Chapter 1 – Introduction

1.0– Introduction to Antibiotic Resistance

Bacterial antibiotic resistance is an issue that has rapidly escalated during the last few decades (13, 14, 49). In today's healthcare system, antibiotics that were once extremely effective, and regularly used, no longer have the ability to fend off life-threatening infections due to high levels of antibiotic resistance. In the natural environment, the development of resistance mechanisms may be employed as survival techniques to protect the bacteria against antibiotics produced by neighboring bacteria, as well as against antibiotics that are self-produced. The frequent and excessive use of antimicrobial agents in the clinical setting has been thought to accelerate this process (13). The issue of resistance has escalated to the point where all families of antibiotics and antimicrobial agents now experience resistance to some extent; even new antibiotics and inhibitors already exhibit some level of resistance (13, 29, 37, 43). It is estimated that, "by the year 2020, the number of deaths due to chronic obstructive pulmonary disease and pneumonia will be exceeded only by chronic heart disease and stroke", a large contributing factor being the increase in drug-resistant organisms, such as penicillin-resistant *Streptococcus pneumoniae* (3).

As the emergence of resistant bacteria increases, new drugs and drug targets must be discovered; however, the creation of new drugs simply cannot keep up with the quickly evolving bacterial species (51). Unfortunately, we have to accept that antibiotic discovery goes hand in hand with antibiotic resistance and these cannot be separated. Consequentially, the pharmaceutical research industry is contemplating new strategies to combat resistant bacteria. These include searching for new antibiotics in the natural

environment, discovering novel antibiotics synthetically, modifying existing antibiotics to restore their activity against resistant bacteria, and searching for compounds that inhibit bacterial resistance mechanisms (51).

1.1 Mechanisms of Resistance

Bacteria have acquired the ability to resist antimicrobial agents in one of two ways: intrinsically, by vertical transfer of favorable mutations to future generations, or by innate mechanisms such as efflux pumps, biofilms, and the outer membrane of Gram negative bacteria, or extrinsically, by acquiring mobile genetic elements, such as transposons and plasmids, that are carrying resistance genes (44). Once the bacteria have acquired the genetic material that encodes modes of resistance, they can establish antibiotic resistance in the cell in multiple ways such as, modification of the antibiotic target, inactivation of the antibiotic, growth as biofilms, and efflux of the antibiotic out of the cell (51).

A new strategy to fight resistant bacteria could be to search for compounds that inhibit bacterial resistance mechanisms (51). The use of a compound that can inhibit one or more of these mechanisms, used in combination with a drug which is no longer effective, can recover the original antibiotic activity (51). A prime example of a drug-inhibitor pair already in use in medical practice is Augmentin®, which is the combination of the β-lactam antibiotic amoxicillin, and the β-lactamase inhibitor clavulanic acid, which is a natural product of *Streptomyces clavuligerus* (9, 34). Although inhibitors of

antibiotic resistance may be created using synthetic methods, a more effective alternative might be to search for inhibitors found in the natural environment. Environmental bacteria in their natural setting must cope with constant exposure to antibiotics, and other toxic molecules, secreted by both themselves and by other species (8). As well, these bacteria must be able to handle encounters with other species that are resistant to their own defense mechanisms (53). As such, there is a likely evolutionary pressure for antibiotic-producing bacteria to develop their own resistance mechanisms, as well as to develop inhibitors of resistance mechanisms (15, 50). With this in consideration, it is possible that these bacteria have already established an ability to challenge resistance in their own environment. One of these strategies may be through the secretion of compounds that inhibit these mechanisms. By isolating these compounds and coupling them with antibiotics which are no longer effective due to high levels of resistance, the antimicrobial activity of these antibiotics could be restored (51).

1.2 Antibiotics and Resistance

Prior to the emergence of resistant bacteria in the clinic, antibiotics of high potency were routinely used to fight off critical infections. Among these antibiotics were the β-lactams, such as penicillin, the macrolides, such as erythromycin, the aminoglycosides, such as kanamycin, and the chloramphenicols, all of which were derived from natural products of bacteria (44). These four groups are the antibiotic focus of this study for multiple reasons. These groups not only represent different chemical

classes of antibiotics, but they include compounds that have well characterized molecular targets and many of them have available crystal structures. Additionally, they are either clinically relevant today, or have been in the past.

1.2.1 – β-lactams

The β-lactam antibiotics were clinically introduced in 1941. They include the penicillins, cephalosporins, carbapenems, and monobactams, and are the most commercially produced family of antibiotics in the world (26). β-lactams are characterized by the presence of a β-lactam ring and act specifically as inhibitors of cell wall synthesis in bacteria (see figure 1.1) (26). The peptidoglycan cell wall is composed of an alternating disaccharide unit of *N*-acetyl-glucosamine and *N*-acetyl muramic acid. The *N*-acetyl muramic acid is modified by a pentapeptide which ends in two D-alanine residues. The final cross-linking of the D-alanine residues is catalyzed by the cell-wall transpepsidase enzymes, which form a peptide bond between them (48). These transpeptidase operate using a catalytic active site serine nucleophile, as a result they readily react with electrophilic β-lactam rings. β-lactam molecules are able to bind to the transpeptidase enzymes and undergo nucleophilic attack by the active site serine. This covalent binding inhibits the formation of the cell wall, eventually causing lysis of the cells, and cell death, due to osmotic pressure (26).

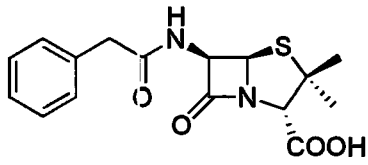
Modification of penicillins has enabled the introduction of new, semi-synthetic antibiotics in response to the high rate of resistance acquisition. These include drugs

such as ampicillin, methicillin and carbenicillin, which can target a broad spectrum of bacteria, unlike the natural penicillin, benzylpenicillin (penicillin G), which can only target Gram positive bacteria (see figure 1.1) (26). These semi-synthetic β-lactams also have been developed to address the problem of resistance.

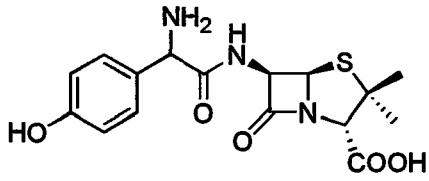
Resistance to β-lactams was first observed in 1940, one year before they were introduced into the clinics, by Abraham and Chain when they discovered the first β-lactamase enzyme, penicillinase in *Escherichia coli* (1). Both Gram positive and Gram negative bacteria express β-lactamase enzymes, which hydrolyze β-lactam antibiotics by cleaving the β-lactam ring (see figure 1.2). There are now well over 340 known β-lactamases which have been categorized into four classes, A, B, C and D (9, 10, 32). Classes A, C and D cleave the β-lactam ring through the action of a serine nucleophile active site and are further classified according to amino acid sequence (see figure 1.2A) (10, 50).

There has been considerable success in discovering inhibitors of class A, C and D β-lactamases (9, 32, 37, 51). For example, the compound clavulanic acid a weak antibiotic, yet it is more important as a strong inhibitor of β-lactamase hydrolysis (28, 32). Other inhibitors such as sulbactam and tazobactam, are also well characterized and can successfully inhibit β-lactamases (see figure 1.3) (9). These inhibitors form an enzyme-Ser-inhibitor complex in the first step of catalysis however it is not able to undergo the second step of hydrolysis and release the enzyme from the complex, thus rendering the β-lactamase enzyme inactive (52). Class B β-lactamases require one or two Zn^{2+} ions bound to their active site and are, therefore, known as metallo-β-lactamases

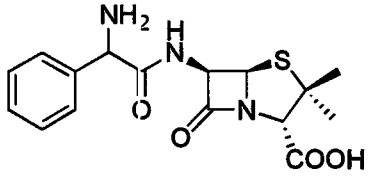
(see figure 1.2B) (10, 50). Recently, Bulgecin A, a potential inhibitor of class B β-lactamases, has been discovered (see figure 1.3); Bulgecin A has been shown to inhibit binuclear zinc-dependant metallo-β-lactamases *in vitro* by chelating the Zn^{2+} active site of the enzyme (36). Even with this discovery, class B β-lactamases present a growing and significant problem, due to both the limited number of promising inhibitors and the ease at which the associated resistance genes are transferred between Gram-negative pathogens (45). Although there has been significant success with the use of previously discovered β-lactamase inhibitors, bacterial resistance to the inhibitors of class A has already been discovered; thus, the search for new inhibitors must continue (2, 9, 37, 40).



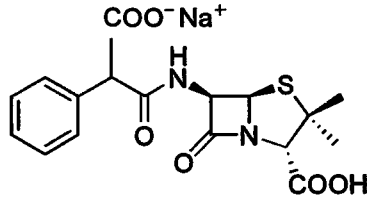
Penicillin G



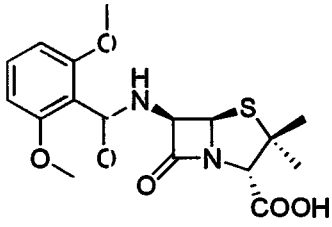
Amoxicillin



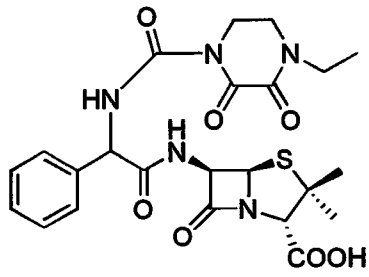
Ampicillin



Carbenicillin



Methicillin



Piperacillin

Figure 1.1. β-lactam antibiotics.

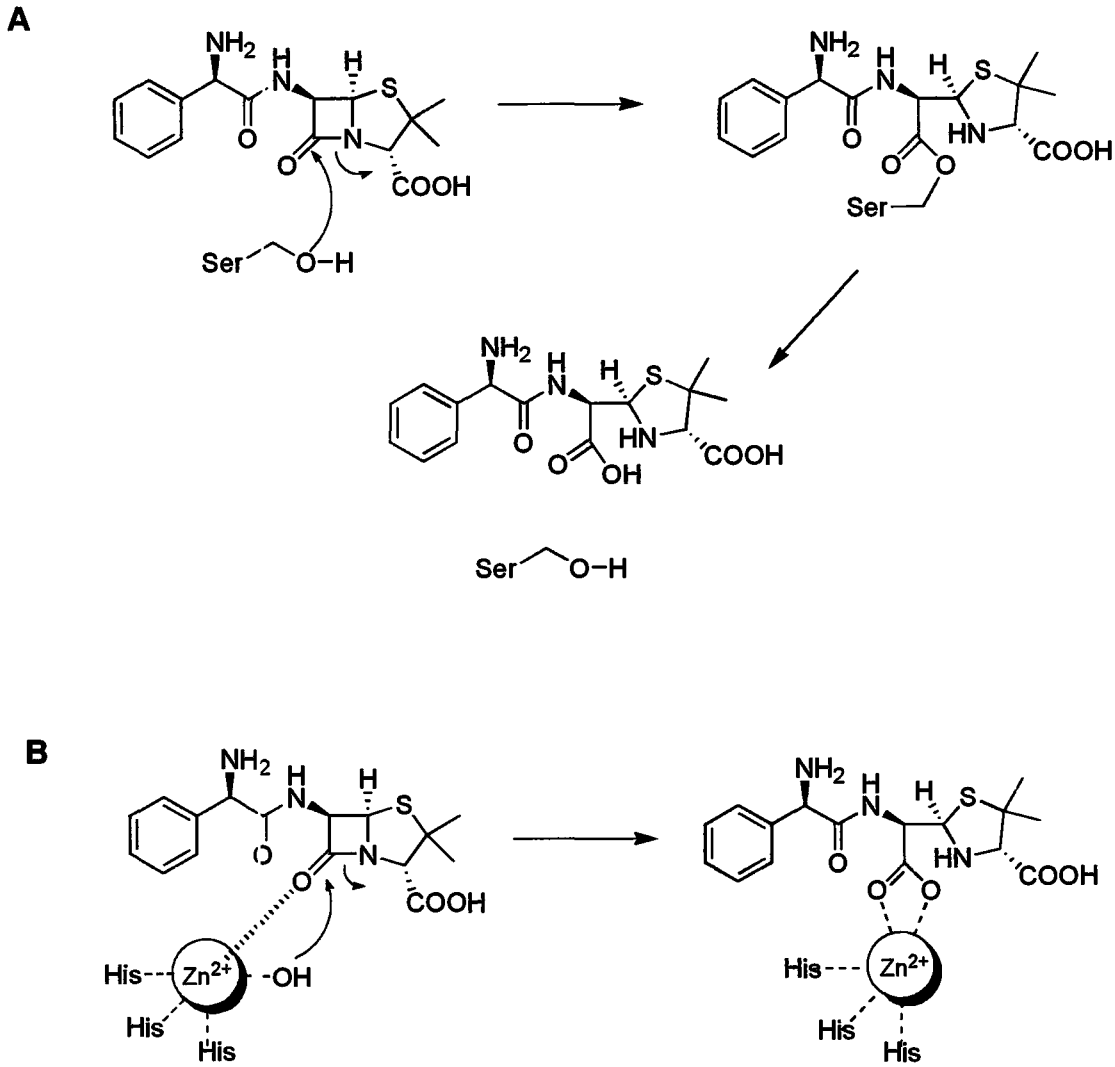
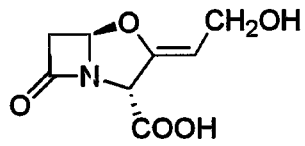
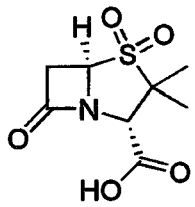


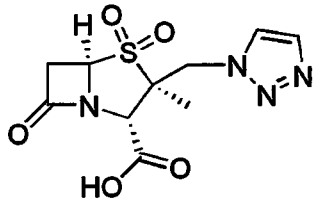
Figure 1.2. Mechanisms of β-lactamases. Schematic shows inactivation of ampicillin, a representative β-lactam antibiotic. **A)** Serine β-lactamases catalyze hydrolysis **B)** Metallo-β-lactamases utilize a zinc ion to cleave the β-lactam ring. (Adapted from Wright, 2005)



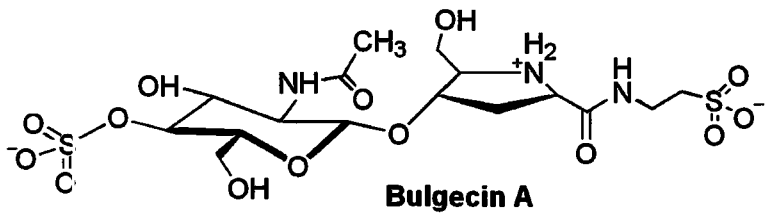
Clavulanic acid



Sulbactam



Tazobactam



Bulgecin A

Figure 1.3. β-lactamase inhibitors. Clavulanic acid, sulbactam and tazobactam inhibit serine β-lactamases and Bulgecin A inhibits binuclear metallo-β-lactamases *in vitro*.

1.2.2 – Macrolides

Similar to the β-lactams, macrolide antibiotics account for a relatively large proportion of the world's total antibiotic production. These antibiotics, such as erythromycin, azithromycin, and telithromycin (see figure 1.4), inhibit protein synthesis at the 50S subunit of the ribosome and are classified by large macrocyclic lactone rings, which are modified by sugar moieties (44). Through the interaction with 23S ribosomal RNA (rRNA), macrolides block the peptide exit tunnel, inhibiting protein elongation, thus allowing for early release of peptidyl-tRNA intermediates (50). As well, this interaction also blocks the assembly of 50S subunits (44).

The mechanism of resistance common to macrolide antibiotics is a base-specific methylation of the 23S rRNA, near or within the antibiotic binding site, preventing the antibiotic from binding to the ribosome (19, 20). For the erythromycin family of antibiotics specifically, this occurs by the activation of a methyltransferase of the erythromycin-resistant methylase family (ERM) (44, 46). Using *S*-adenosylmethionine as a methyl group source, the ERMs are able to catalyze the mono or dimethylation of the adenine at the N6 position of the rRNA (20). Hajduk *et al.* have identified a group of ERM inhibitors which bind to the enzyme and inhibit the methylation of rRNA (20). This group of inhibitors include synthetic compounds, such as 2-aminobenzthiazole and 7-azaindole (see figure 1.5) (20). As well, macrolide antibiotics can be attacked by erythromycin esterases, such as EreA and EreB, which cleave the macrocycle ester bond (50).

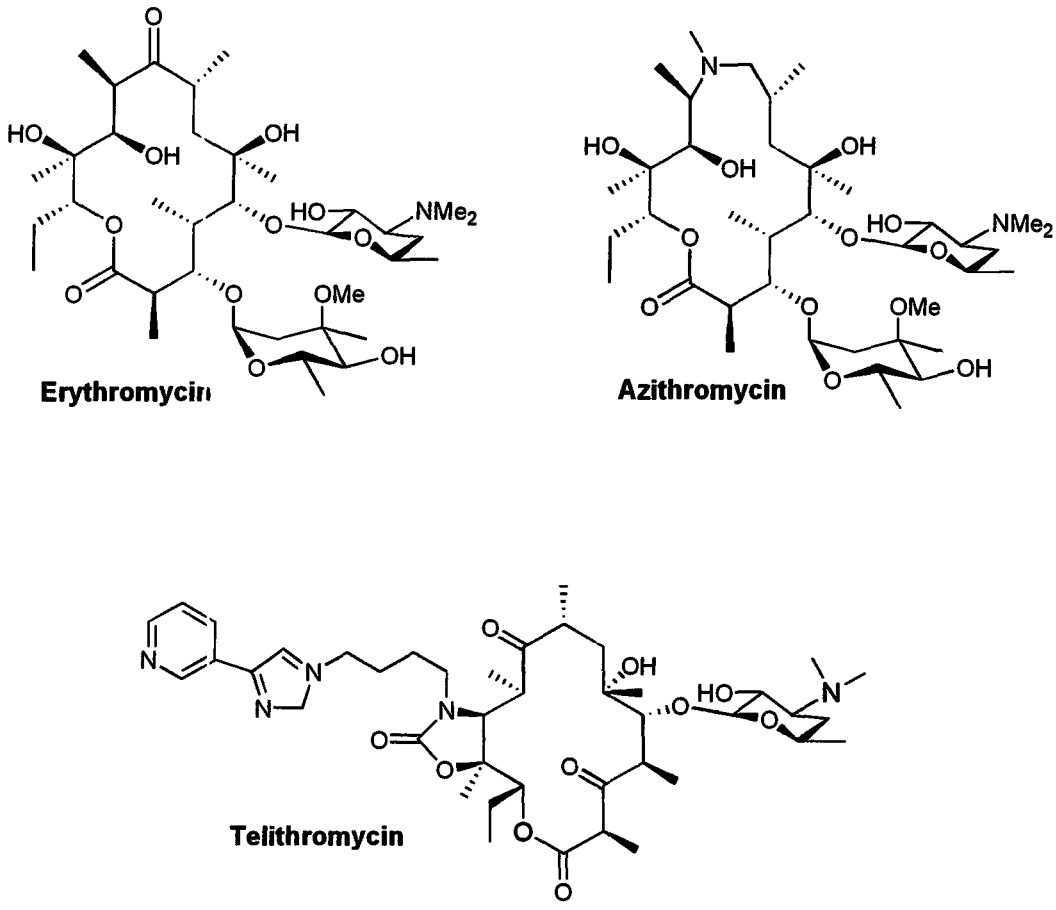
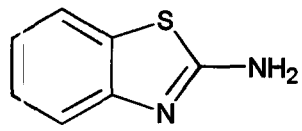
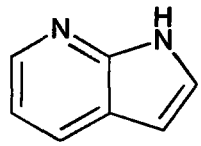


Figure 1.4. Macrolide antibiotics.



2-aminobenthiazole



7-azaindole

Figure 1.5. Erm inhibitors.

1.2.3 – Aminoglycosides

The aminoglycoside family of antibiotics consists of amino sugars bonded by glycosidic linkages and include an aminocyclitol ring (26). Similar to the macrolide antibiotics, aminoglycosides also inhibit protein synthesis, however they act on the 30S subunit of the ribosome, as opposed to the 50S subunit for macrolides. They specifically target the 16S rRNA at the A site of the 30S subunit. Binding at this site interferes with cognate codon-anticodon recognition and the proofreading process, resulting in the misincorporation of amino acids into the growing polypeptide chain, producing aberrant proteins (6, 23, 27, 30, 39, 44). Aminoglycosides, such as streptomycin and kanamycin (see figure 1.6), were once highly used to fight infections caused by Gram negative bacteria, however, as a result of negative side-effects, as well as the rise of antibiotic resistance, they are no longer as common in clinical practice (26).

In order to achieve resistance to these antibiotics, bacteria have acquired the ability to chemically modify them (49). By producing a variety of enzymes, including *O*-adenyltransferases, *O*-phosphotransferases, and *N*-acetyltransferases, bacteria can successfully inactivate aminoglycoside antibiotics (29). These enzymes take advantage of exposed hydroxyl and/or amino groups and covalently modify them in order to inactivate the antibiotic (50). A large spectrum of antibiotics can be modified at the 3'-hydroxyl group by multiple classes of aminoglycoside phosphotransferase (APH) enzymes, specifically APH(3')s, which are expressed in both Gram negative and Gram positive bacteria (41); type I and II APHs are expressed in Gram negative bacteria and type III APHs are expressed in Gram positive bacteria (29, 42). APH(3')-IIIa, one of the most common APHs, is well characterized and has also been shown to have significant structural homology to other phosphotransferases that attack aminoglycosides at the 3'-hydroxyl (see figure 1.7) (29), (41). APH(3')-IIIa confers resistance to a wide range of aminoglycosides, with the exception of tobramycin and gentamicin, which lack the 3'-hydroxyl target. These two antibiotics are commonly targeted by APH(2") (50). It has been shown that inhibition of aminoglycoside-modifying enzymes can be achieved using protein kinase inhibitors. Kinase inhibitors that are especially effective are those that belong to the isoquinoline-sulphonamide group which competitively bind to the ATP-binding site of the resistance enzymes (see figure 1.8) (12).

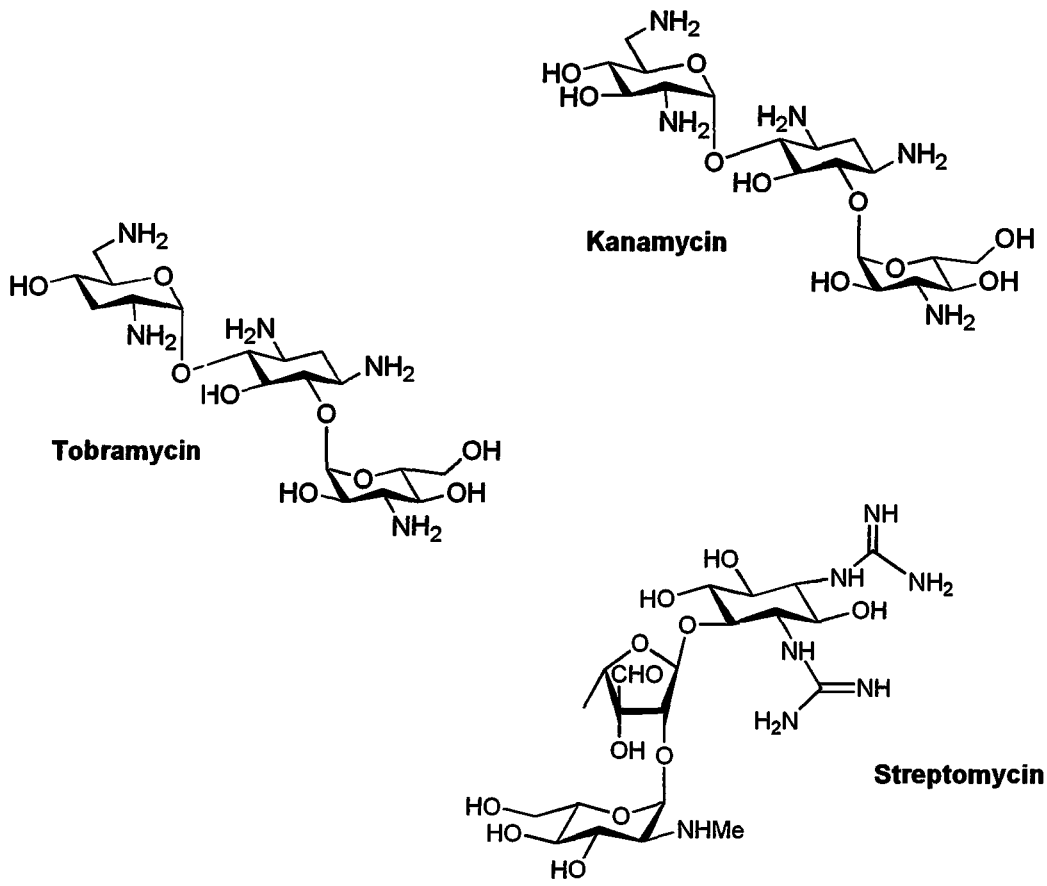


Figure 1.6. Aminoglycoside antibiotics.

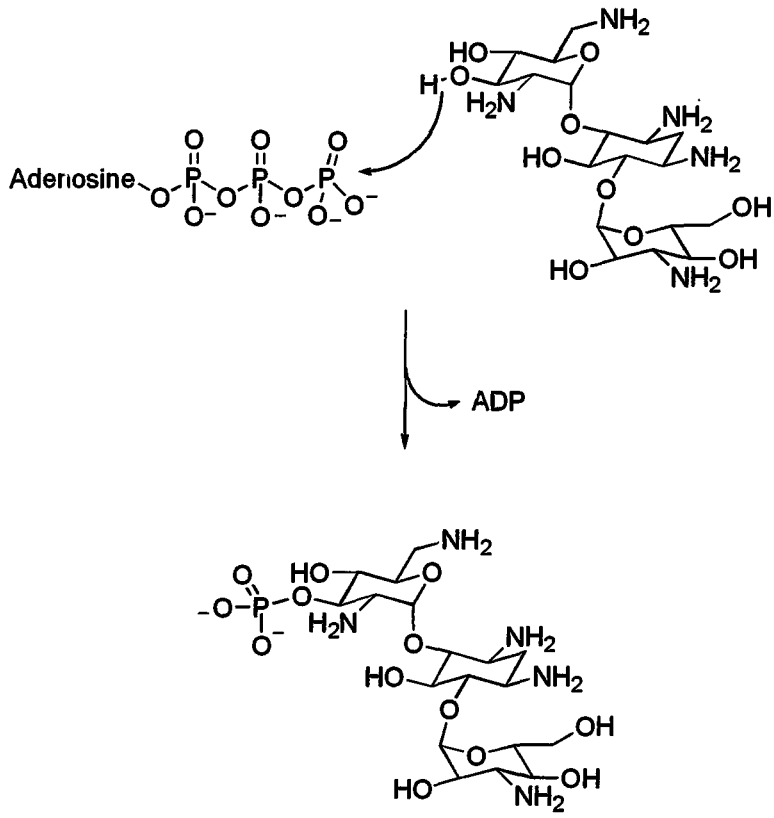


Figure 1.7. Mechanism of APH(3')-IIIa. The inactivation of kanamycin by phosphorylation, as catalyzed by APH(3')-IIIa. (Adapted from Wright 2005)

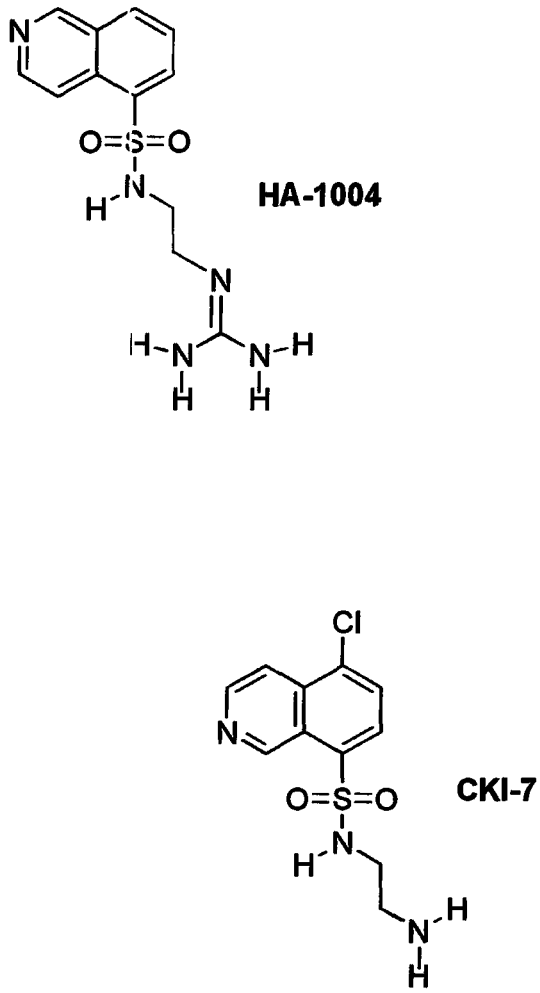


Figure 1.8. APH inhibitors. Inhibitors N-(2-guanidinoethyl)-5-isoquinolinesulfonamide (HA-1004), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), and N-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonimide (CKI-7).

1.2.4 – Chloramphenicols

Chloramphenicols are relatively small, bacteriostatic drugs which act on peptidyl transferase centers of bacterial ribosomes (33). They bind to the 50S subunit of the ribosome at the peptidyltransferase center, thereby occluding the ribosomal A site and blocking aminoacyl-tRNA interaction at the ribosome (44).

Resistance to chloramphenicol was reported in the early 1950's in *E. coli* and is initiated by chloramphenicol acetyltransferases (CATs), which *O*-acetylate the primary alcohol (50). A histidine residue in the active site of CATIII acts as an active site base in order to deprotonate the nucleophilic hydroxyl group of chloramphenicol (see figure 1.9) (50). These enzymes transfer the acetyl group of acetyl-CoA to the hydroxyl group of the antibiotic, resulting in acetylchloramphenicol, which is unable to bind to the ribosome and subsequently block protein synthesis due to its structure (31). In Gram negative bacteria, CATs are categorized into three groups: I, II, and III. CATs found in Gram positive bacteria are classified by homology to different CAT sources. An example is the CAT_{pC194} group, which has homology to the CAT gene found on plasmid pC194 in *Staphylococcus aureus.*

So far there has only been minimal success of finding inhibitors of CATs in Gram negative bacteria, specifically inhibition of CAT_{II} by thiol-reactive reagents, such as 5,5-'dithiobis-(2-nitrobenzoic acid) (DTNB) and 4,4'-dithiodipyridine (DTDP) (see figure 1.10) due to the presence of a reactive Cys-31 residue within the chloramphenicol-binding site (31). Successful inhibitors of CATs in Gram positive bacteria, however, have yet to be discovered.

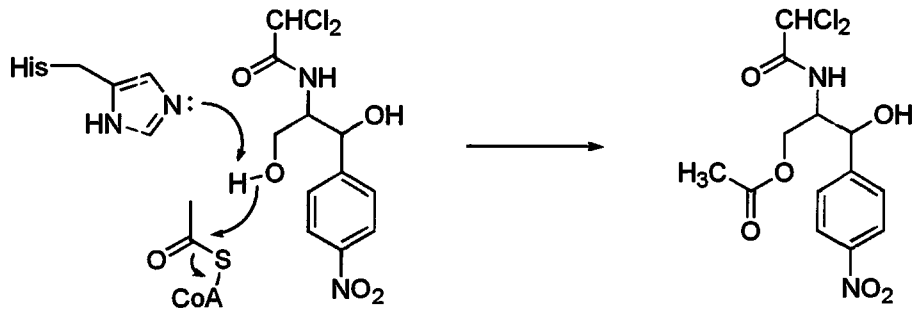


Figure 1.9 . Mechanism of CAT. Inactivation of chloramphenicol by chloramphenicol acetyltransferases (Adapted from Wright 2005).

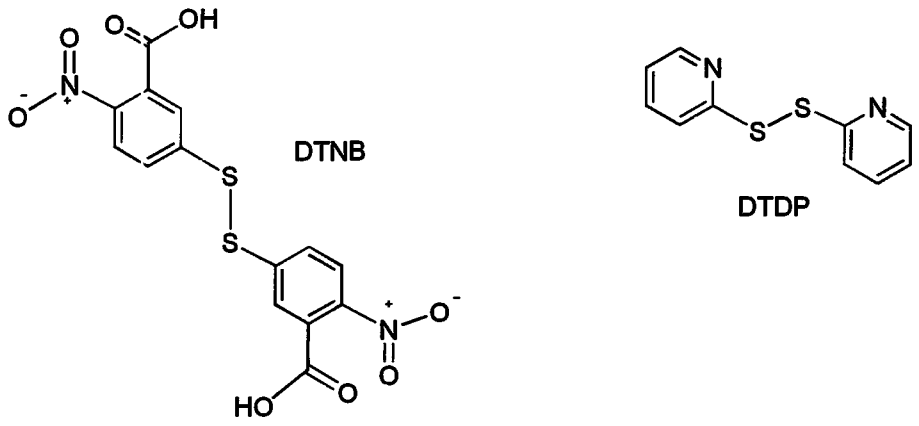


Figure 1.10. CAT inhibitors. 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and 4,4'-dithiodipyridine (DTDP) inhibit chloramphenicol acetyltransferases of type CAT_{III}.

1.3 – Research Objectives

Currently there are various approaches to the problem of antibiotic resistance which include, modifying existing antibiotic scaffolds, which has generally been the main approach since 1970, identifying new antibiotics by either inventing new antibiotics synthetically or by searching the natural environment for microbes which produce new antibiotics, or by rescuing antibiotic activity by finding new inhibitors of antibiotic resistance. The β-lactam, macrolide, chloramphenicol, and aminoglycoside families of antibiotics, as well as the resistance mechanisms working against them, are the central focus of this research. These families not only represent well-characterized groups of antibiotics, but they are, or at least were, clinically important due to their powerful ability to fight against critical infections. It is vital that a solution is found that can once again facilitate the use of these drugs in clinical practice. Therefore by searching for compounds that inhibit resistance mechanisms in bacteria we may be on the path to revitalizing the use of these well-characterized antibiotics.

A search for novel inhibitors of resistance mechanisms is an ideal way to make use of drugs already available and optimized for clinical usage. The focus of this research is to develop expression systems, in Gram positive and Gram negative bacteria, which contain inducible antibiotic resistance genes. Once operational, these systems will then be used to screen libraries of natural product extracts, as well as commercial compound libraries, in order to search for novel inhibitors of antibiotic resistance.

The main goals of this project include:

1) Develop expression vectors that contain inducible resistance genes.

2) Introduce these vectors into model Gram positive and Gram negative strains.

3) Optimize screening conditions for these strains for future uses such as:

a) Screening libraries of commercial compounds

b) Screening libraries of natural products from natural product extracts

Chapter 2 – Materials and Methods

2.0 - Bacterial Strains and Growth Conditions

The wildtype strain of *B. subtilis*, BGSC 1A1, a tryptophan auxotroph due to a *trpC2* mutation, was used for expression of the integrated resistance gene product. The *B. subtilis* strain, EB648, which has an inactivated *amyE* gene, was used as a negative control for comparison of integration into the *amyE* locus. All *B. subtilis* strains were grown overnight either as a liquid culture in Luria Broth (LB) purchased from Bioshop (Burlington, ON), at 30°C and 250 rpm, or on solid LB (Bioshop, Burlington, ON) agar plates at 30 °C. Integration of a resistance gene into the bacterial chromosome was selected on LB agar plates supplemented with 4% xylose as well as 5 μg/mL kanamycin purchased from Bioshop Canada, Ltd. (Burlington, ON), 1 μg/mL ampicillin purchased from Bioshop Canada, Ltd. Burlington, ON), 5 μg/mL chloramphenicol purchased from EM Science (Gibbstown, NJ), or with 0.5 μg/mL erythromycin purchased from Sigma-Aldrich (St. Louis, MO). Minimal inhibitory concentration (MIC) assays of the *B. subtilis* strains were performed using cationic-adjusted Muller Hinton Broth purchased from BD Biosciences (Mississauga, ON).

E. coli Top10 cells were used for all cloning purposes and *E. coli* Tuner (DE3) cells from Novagen (San Diego, CA) were used for expression of resistance genes in pETcoco plasmids. Tuner cells are *E. coli* BL21(DE3) cells that contain a *lacZY* deletion mutation. All *E. coli* strains were grown overnight either as a liquid culture in Luria Broth (LB) at 37°C and 250 rpm, or on solid LB agar plates at 37°C. Plates and cultures were supplemented with 50 μg/mL kanamycin, 100 μg/mL ampicillin, or with 12.5 μg/mL chloramphenicol, for plasmid selection. Minimal inhibitory concentration (MIC)

assays of the *E. coli* strains were performed using cationic-adjusted Muller Hinton Broth (BD Biosciences, Mississauga, ON).

2.1 – Recombinant DNA Methods and Gene Amplification

DNA analysis was performed using 1% Tris-acetate-ethylenediamine-tetraacetic acid (TAE) agarose gel electrophoresis stained with Sybr Safe DNA gel strain from Invitrogen (Burlington, ON). DNA fragment size was compared to the Generuler 1 KB DNA ladder purchased from Fermentas (Burlington, ON). Plasmid DNA was extracted from *E. coli* strains using Qiagen QIAprep Spin Miniprep Kit (Qiagen Inc., Mississauga, ON) following the protocol outlined by the manufacturer. Plasmids and amplified PCR products were gel purified using the QIAEX II Gel Extraction Kit (Qiagen Inc. Mississauga, ON) following the gel extraction protocol outlined by the manufacturer. All DNA digests were performed using restriction enzymes from New England Biolabs Inc. (Ipswich, MA).

Gene amplification was achieved by Polymerase Chain Reaction (PCR). Plasmid DNA was used as a template for the amplification of resistance genes to be cloned into pSWEET plasmids from Dr. Eric D. Brown's lab, McMaster University, Hamilton, ON (5), and chromosomal DNA was used as a template in amplification of resistance genes to be cloned into pETcoco plasmids, as well as verification of gene integration into the *B. subtilis* chromosome. Reactions were prepared using 1x DNA Polymerase buffer containing 75 mM Tris HCl pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, and 20 mM $(NH_4)_2SO_4$

(Biotools Inc., Edmonton, AB) mixed with a 50x dilution of purified plasmid template DNA, and primers at a concentration of 0.8 μM which were synthesized at the MOBIX Central facility (McMaster University, Hamilton, ON). Each PCR program was customized for each primer set in regards to annealing temperature, purpose of the amplification, and occasionally the magnesium concentration.

For PCR where plasmid DNA was the template, each program began with a 3 minute initial denaturation at 95 °C, and then consisted of 30 cycles of the following: denaturation at 95°C for 1 min, annealing at 5 degrees lower than the melting temperature of the primers for 1 minute, and elongation at 72 °C for 1 minute. At the end of the 30 cycles the reactions were held at 72 °C for 10 minutes. If the gene to be amplified was greater than 1kb in size then the elongation time was increased by 1 minute per 1kb.

For PCR where chromosomal DNA was the template, each program began with a 6 minute initial denaturation at 95 °C, and then consisted of 30 cycles of the following: denaturation at 95°C for 1 min, annealing at 5 degrees lower than the melting temperature of the primers for 1 minute, and elongation at 72 °C for 1 minute. At the end of the 30 cycles the reactions were held at 72 °C for 10 minutes. If the gene to be amplified was greater than 1kb in size then the elongation time was increased by 1 minute per 1kb. Chromosomal DNA was isolated by picking an individual colony and inoculating the PCR mixture directly in the reaction tube.

Colony PCR was performed by isolating a colony, originating from a transformation result, and inoculating the PCR sample directly in the PCR tube. Since colony PCR is typically used for *E. coli,* it had to be optimized for wildtype *B. subtilis*:

The first cycle of the PCR was extended to 10 minutes at 95°C, instead of 5 minutes which is typical for colony PCR with *E. coli*, due to the thick peptidoglycan layer characteristic of Gram positive bacteria. Instagene Matrix (Bio-Rad) was also used to prep genomic DNA for use in PCR reactions; it consists of a Chelex matrix which binds to cell lysis products which would normally interfere with a PCR reaction (Bio-Rad Laboratories, Hercules, CA). Once the matrix is removed through centrifugation, the supernatant is used directly in the PCR reaction. A colony is first resuspended in water and then boiled to lyse the cells, the instagene matrix is added and the sample is incubated at 56°C for 30 minutes, then at 100°C for 8 minutes, before precipitating the resin with a microcentrifuge.

Plasmid pSWEET

Four genes were amplified for insertion into the pSWEET-*bgaB* vector from Dr. Eric D. Brown's lab, McMaster University, Hamilton, ON (5) (see table 1). The *ermB* gene, amplified from plasmid pUC19-*ermB* (Dr. Gerry D. Wright's lab, McMaster University, Hamilton, ON), encodes for the erythromycin-resistant methylase from *S. aureus* (7, 47). The pUC19-*ermB* plasmid DNA was isolated from the *E. coli* strain BAS901. This *ermB* gene was first identified in *S. aureus* (7, 17, 25, 47). The *bla* gene, amplified from plasmid pETSAC-G1 (Dr. Gerry D. Wright's lab, McMaster University, Hamilton, ON), encodes for a TEM-1 β-lactamase, first isolated from the *E. coli* plasmid pBR322 (38). The *aph(3')- IIIa* gene, also amplified from plasmid pETSAC-G1, encodes for the enterococcal kanamycin kinase from *Enterococcus faecalis* (29). The

vector pETSAC-G1 was isolated from the *E. coli* strain BL21(DE3). Finally, the *cat* gene, amplified from plasmid pSWEET-*bgaB,* encodes for the chloramphenicol acetyltransferase of type CAT_{pC194} due to the fact that it originated from the plasmid pC194 in *S. aureus* (22). These four genes were amplified by PCR, using primers designed with recognition sites for *Pac*I and *Nhe*I incorporated in the primer sequences (see table 2).

Gene		Parent plasmid	Origin	Origin of parent plasmid
aph(3')-IIIa	aminoglycoside phosphotransferase type IIIa	pETSAC-G1	*E. faecalis*	*E. coli,* strain BL21(DE3)
ermB	erythromycin-resistant methylase type B	pUC19-*ermB*	*S. aureus*	*E. coli,* strain BAS901
bla	β-lactamase	pETSAC-G1	*E. coli,* pBR322	*E. coli,* strain BL21(DE3)
cat	chloramphenicol acetyltransferase	pSWEET-*bgaB*	*S. aureus,* pC194	*E. coli* strain Top10

Table 1. Origin of resistance genes. Genes *bla, aph(3')IIIa, cat,* and *ermB,* were cloned into pSWEET-*ΔbgaBcat* (7, 17, 22, 25, 29, 38, 47).

Plasmid pETcoco

The resistance genes *bla* and *aph(3')-IIIa* were PCR amplified from previously constructed plasmids, pSWEET-*blaΔcat* and pSWEET-*aph(3')-IIIaΔcat*, respectively. These genes were amplified using oligonucleotides that contain sites for restriction enzymes *Nhe*I and *Hind*III (see table 2 for primers). PCR products were run on a 1% TAE agarose gel and gel extracted using the QIAEX II Gel Extraction kit (Qiagen Inc., Mississauga, ON) and digested with *Nhe*I and *Hind*III for 2 hours at 37°C. The digested PCR products were then purified by being run again on a 1% TAE agarose gel and gel extracted using the QIAEX II Extraction kit.

Primers			
Mutation			**5'- 3' Quikchange® Mutagenesis Primers 5'-3'** (*Mfe*I)
New *Mfe*I site		forward reverse	CGGCATTATCTCATACAATTGTTATAAAAGCCAGTC GACTGGCTTTTATAACAATTGTATGAGATAATGCCG
Vector			**Quickchange Primers 5'-3'** (ribosome binding site)
pSWEET- *aph(3')-IIIaΔcat*		forward reverse	GCGTTAACAAAGTGGTTTAATTAACATTAGGAAGGAGCGTTTCTTTAAATG GCTAAAATGAGAATATCACCGG CCGGTGATATTCTCATTTTAGCCATTTAAAGAAACGCTCCTTCCTAATGTT AATTAAACCACTTTGTTAACGC
pSWEET-*blaΔcat*		forward reverse	GCGTTAACAAAGTGGTTTAATTAACATTAGGAAGGAGCGTTTCTTTAAATG AGTATTCAACATTTCCGTGTCGCCC GGGCGACACGGAAATGTTGAATACTCATTTAAAGAAACGCTCCTTCCTAA TGTTAATTAAACCACTTTGTTAACGC
pSWEET-*cat*		forward reverse	GCGTTAACAAAGTGGTTTAATTAACATTAGGAAGGAGCGTTTCTTTAAATG AACTTTAATAAAATTGATTTAGACAATTGG CCAATTGTCTAAATCAATTTTATTAAAGTTCATTTAAAGAAACGCTCCTTCC TAATGTTAATTAAACCACTTTGTTAACGC
pSWEET-*ermBΔcat*		forward reverse	GCGTTAACAAAGTGGTTTAATTAACATTAGGAAGGAGCGTTTCTTTAAATG AACAAAAATATAAAATATTCTC GAGAATATTTTATATTTTTGTTCATTTAAAGAAACGCTCCTTCCTAATGTTA ATTAAACCACTTTGTTAACGC
Vector			**Sequencing Primers 5'-3'**
pSWEET-*bgaB*		forward	CGATGCGTCCGGCGTAGAG
pSWEET-*bgaBΔcat*		forward reverse	GAGGAAATAACATGGCTCAATCTCATTCTAGTTCA GACACCAGACCAACTGGTAATGGTAGCGA
pETcoco-1		forward reverse	GCATCACCATCACCATCACGG GAGCGAGTTCTGGCTGGCTTG
Vector	**Gene**		**PCR Primers 5'-3'** (*Nhe*I, *Hind*III, *Pac*I)
pUC19-*erm*	*ermB*	forward reverse	GGTTAATTAAATGAACAAAAATATAAAATATTCTCAAAACTTTTTAACG GGCTAGCGGATCCTTATTTCCTCCCGTTAAATAATAGATAACTATTAAAAA TAG
pETSAC-G1	*bla*	forward reverse	GGTTAATTAAATGAGTATTCAACATTTCCGTGTCGCCC GGCTAGCGGATCCGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAG
pETSAC-G1	*aph(3')-IIIa*	forward reverse	GGTTAATTAAATGGCTAAAATGAGAATATCACCGGAATTGAAAAAAC GGCTAGCGGATCCCTAAAACAATTCATCCAGTAAAATATAATATTTTATTTT CTCCC
pSWEET-*bgaB*	*cat*	forward reverse	GGTTAATTAAATGAACTTTAATAAAATTGATTTAGACAATTGGAAGAG GGCTAGCGGATCCTTATAAAAGCCAGTCATTAGGCCTATCTGAC
chromosome	*amyE*	forward reverse	GACTCCGAAGTAAGTCTTCAAAAAATCAAATAAG CATCATTGATGGTTTCTTTCGGTAAGTCC
pSWEET	*amyE* region	forward reverse	GTCTGCCCGTATTTCGCGTAAGG GTAGTAGGTTGAGGCCGTTGAGCAC
pSWEET-*aphΔcat*	*aph(3')-IIIa*	forward reverse	GGCTAGCATGGCTAAAATGAGAATATCACCGGAATTGAAAAAAC GAAGCTTCTAAAACAATTCATCCAGTAAAATATAATATTTTATTTTCTCCC
pSWEET-*blaΔcat*	*bla*	forward reverse	GGCTAGCATGAGTATTCAACATTTCCGTGTCGCCC GAAGCTTTTACCAATGCTTAATCAGTGAGGCACCTATCTCAG

Table 2. Primers designed for Quikchange® mutagenesis, sequencing reactions, and PCR amplification. Primers were synthesized by MOBIX Central facility (McMaster University, Hamilton).

2.2 – Plasmid Constructions

Plasmid pSWEET

The pSWEET-*bgaB* plasmid was isolated from the *E. coli* strain Novablue (Novagen, Gibbstown, NJ), which was grown in LB media with 100 μg/mL ampicillin (Bioshop, Burlington, ON). The chloramphenicol resistance cassette (Cm^R) cassette was eliminated from the plasmid by the introduction of a second *Mfe*I restriction site near the 3' end of the gene using Polymerase Chain Reaction (PCR) and Quikchange® site directed mutagenesis protocols (Stratagene, La Jolla, CA) (see table 2 for primers). An *Mfe*I site already existed at the 5' end of the Cm^R cassette, so the incorporation of this second *Mfe*I restriction site allowed for the elimination of the Cm^R cassette by a single restriction enzyme digest with *Mfe*I for 2 hours at 37°. Once digested with *Mfe*I, the linearized pSWEET-*bgaBΔcat* vector was ligated back together using T4 DNA ligase. The ligation mix was then de-salted using the QIAEX II gel extraction transformed into *E. coli* TOP10 cells by electroporation, and grown in LB with 100 μg/mL ampicillin. Transformants were screened for proper fusion using restriction mapping, as well as sequencing of the fused region by the MOBIX Central facility.

Amplified resistance genes, as well as the pSWEET-*bgaBΔcat* vector, were digested with restriction enzymes *Nhe*I and *Pac*I at 37°C for 2 hours in order to excise the *bgaB* gene, a 2019bp heat-stable β-galactosidase gene from *Bacillus stearothermophilus,* from the pSWEET-*bgaBΔcat* vector, and to create regions of unpaired nucleotides at the 3' ends, on the PCR products (5). The digested resistance genes were then ligated with

the linear pSWEET-*ΔbgaBcat* vector using T4 DNA ligase. The ligation mix was then de-salted using the QIAEX II gel extraction kit, transformed into *E. coli* TOP10 cells by electroporation, and grown in LB with 100 μg/mL ampicillin. Transformants were screened for proper insertion of the resistance genes using restriction mapping, as well as sequencing of the insertion site by the MOBIX Central facility.

Plasmid pETcoco

The vector pETcoco-1 was transformed into *E. coli* Top10 cells, via electroporation, and plated on LB agar plates with 12.5 μg/mL chloramphenicol (Cm). Cultures were grown up in 50mL of liquid LB media supplemented with 12.5 μg/mL Cm, and 0.01% arabinose to induce medium-copy state for cloning purposes. The plasmid was then isolated, digested with *Nhe*I and *Hind*III for 2 hours, run on a 1% agarose gel, and gel extracted using the QIAEX II Gel Extraction Kit. Amplified resistance genes were also digested with restriction enzymes *Nhe*I and *Hind*III at 37°C for 2 hours to create overhangs on the PCR products. The digested resistance genes were then ligated with the linear pETcoco vector using T4 DNA ligase. The ligation mix was then de-salted using the QIAEX II gel extraction kit, transformed into *E. coli* TOP10 cells by electroporation, and grown in LB with 100 μg/mL ampicillin. Transformants were screened for proper insertion of the resistance genes using restriction mapping as well as sequencing of the insertion site by the MOBIX Central facility.

2.3 – Transformations

Before transforming *B. subtilis*, all pSWEET constructs were digested with *Pst*I in order to linearize the vector, with the exception of pSWEET-*blaΔcat* which was digested with *Apa*I and *Aat*II due to the presence of a *Pst*I recognition site in the β-lactamase gene (*bla*). It was important to transform linear DNA since linearized DNA is taken up into the cells with greater efficiency than circular DNA in *B. subtilis* (21).

Before *B. subtilis* transformations began, the working concentrations of each antibiotic had to be determined. This was completed by streaking out the *B. subtilis* strain, BS1A1, on LB agar plates with different concentrations of drug, as well as setting up simulated transformations. Each simulated transformation consisted of $1/10^{th}$ of the normal plated volume of a *B. subtilis* transformation. The normal transformation volume of 201 µL -220 µL consisted of 100 µL of *B. subtilis* BS1A1 wildtype cells, 100 µL of SPII media (containing 2% xylose), and between 1 µL -20 µL (approximately 0.1µg/uL) of linear DNA which is according to protocols for *B. subtilis* transformations (21). In the simulated transformations, 20 µL of ddH_2O was added to the 200 µL mixture of cells and media instead of DNA. Of the simulated transformations, 20µL was plated onto the LB agar plates of different drug concentrations and 2% xylose. The plates were then incubated at 37°C for 18 hours. Competent *B. subtilis* cells were successfully grown and transformed with the various pSWEET constructs, according to a protocol for optimal transformations of *B. subtilis* in the laboratory environment (21).

All *E. coli* cells were made competent using an electroporator, and transformed, according to the instructions provided by the manufacturer of the electroporation

apparatus (Micropulser Electroporation Apparatus – Operating Instructions and Applications Guide, Section 5, "High Efficiency Electrotransformation of *E. coli*", Bio-Rad Laboratories, Hercules, CA).

2.4 – Minimal Inhibitory Concentrations and Screen Optimization

Minimal inhibitory concentration (MIC) values for *E. coli* cells were obtained according to the Clinical and Laboratory Standards Institute (CLSI) protocols for assaying MIC values. MIC assays were performed in 96 well, round bottom plates, to replicate screening conditions. Each plate contained a row of wells without drug as a growth control in addition to a row of wells without inoculum as a sterility control. All 96 well plates were incubated overnight at 37°C, shaken in a spectrophotometer (Molecular Devices – Spectra Max Plus) for 5 minutes and then read at a wavelength of 600nm. Screening conditions were optimized for a library of chemical compounds, that was suspended in dimethyl sulfoxide (DMSO), by testing for fluctuations in cell growth in the presence of various concentrations of DMSO. As well, different inoculum concentrations, obtained by dilution in MHB media, were employed to determine a suitable endpoint for screening.

Chapter 3 – Results

3.0 - The pSWEET System

To accurately express recombinant resistance genes of interest, a system that delivers controlled expression by an inducible promoter, allows for single-copy expression, is tightly regulated, and contains no additional resistance cassettes, is desired. Following these criteria, the vector pSWEET-*bgaB* was chosen for use in the wildtype strain of *Bacillus subtilis*, BS1A1, a model Gram positive bacterial strain.

The expression vector pSWEET-*bgaB* (see figure 3.1) contains the xylose regulatory system (5). For xylose to be utilized by *B. subtilis,* the production of xylose isomerase (XylA) and xylulose kinase (XylB) is required (5). These genes are included in the xylose operon and are controlled at the level of transcription by intergenic *xyl* operator sequences (5). The *xyl* operator sequences contain the inducible *xylA* promoter, which is repressed when bound by the *xylR* repressor or by catabolite repression by the cis-acting catabolite responsive element (CRE), which is also located in the *xylA* coding sequence (5). The xylose regulatory system can be integrated into the *B. subtilis* chromosome at the *amyE* locus by double recombination of the linear vector at *amyE* integration sites (5).

The xylose promoter-operator system is ideal for expression of resistance genes since it is well-characterized, easily induced, and has higher expression than other systems used in *B. subtilis,* such as the *spac* system (5). It is also tightly regulated in that there are very low levels of induction in the absence of an inducer (5). The only drawback is that pSWEET-*bgaB* contains a chloramphenicol resistance cassette (Cm^R), which needed to be removed prior to use.

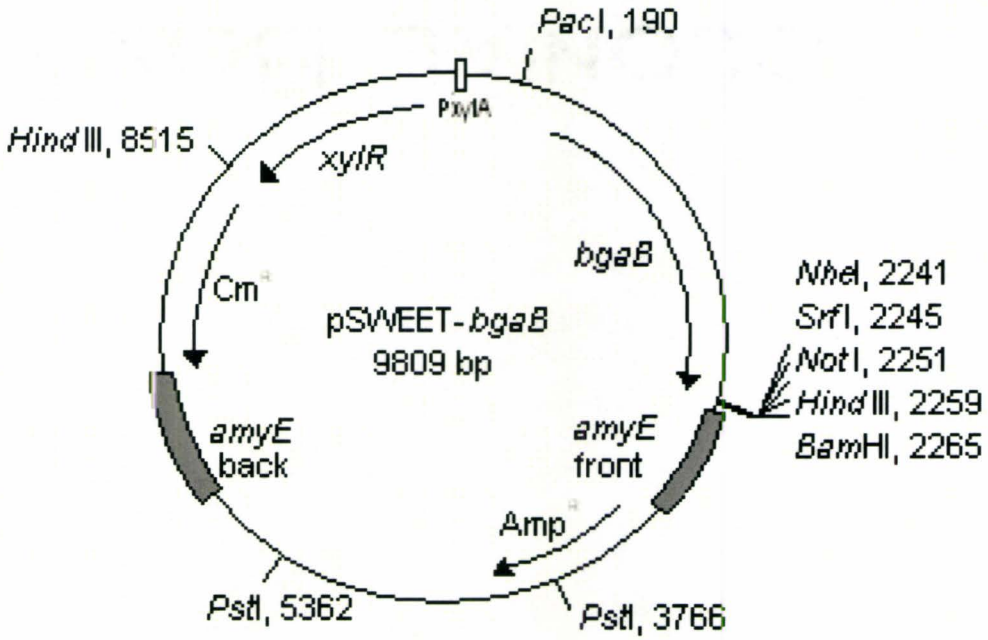


Figure 3.1. Expression vector pSWEET-*bgaB*. Map of pSWEET-*bgaB* which contains ampicillin and chloramphenicol cassettes, as well as a β-galactosidase gene for selection purposes, an *E. coli* origin of replication for cloning procedures, an MCS, the xylose operon, and *amyE* segments for integration into the *B. subtilis* chromosome by double recombination (5). (Adapted from Bhavsar 2001)

3.1 – Amplification of Resistance Genes for pSWEET and Vector Construction

The pSWEET-*bgaB* plasmid was isolated from the *E. coli* strain Novablue, and the Cm^R cassette was removed by introducing a flanking *Mfe*I site at the 3' end of the *cat* gene, utilizing Quikchange® site directed mutagenesis protocols (see table 2 for primers). The presence of a pre-existing *Mfe*I site at the 5' end of the *cat* gene allowed for the elimination of the *cat* gene by a single restriction enzyme digest with *Mfe*I. Once digested with *Mfe*I, the two sticky ends of the linearized pSWEET-*bgaBΔcat* vector were ligated together using T4 DNA ligase. The correct construction of the vector was verified using restriction mapping and DNA sequencing by MOBIX (see figure 3.2).

Four resistance genes, *ermB, bla, aph(3')-IIIa* and *cat,* were PCR amplified from template plasmids (see table 1), using oligonucleotides that were designed with recognition sites for *Pac*I and *Nhe*I incorporated in the primer sequences. The amplified resistance genes, as well as the pSWEET-*bgaBΔcat* vector, were digested with *Nhe*I and *Pac*I and purified on a 1% TAE agarose gel electrophoresis. The digested resistance genes were then ligated with the linear pSWEET-*ΔbgaBcat* vector. Unsuccessful ligation reactions were approached by varying different components of the reaction, as well as the transformation. These include altering DNA concentrations and the ratios of vector to insert, using different quantities of T4 DNA ligase, using newly purchased ligase and ligase buffer, making new batches of electrocompetent *E. coli* Top10 cells, using different gel-extraction methods, de-salting the ligation mix using the Qiaex II® (Qiagen) DNA desalting protocol before transformation via electroporation, adding polyethylene glycol, using different ligation incubation times and temperatures, using different kinds

of ligases, and using different transformation techniques, therefore using different types of competent *E. coli* cells, and different electrocuvette sizes. The ligation reactions were transformed into *E. coli* Top10 cells and transformants were selected on LB plates with 100 μg/ml ampicillin. Once successfully ligated together, the correct fusion of DNA fragments was confirmed by restriction mapping, as well as by sequencing at MOBIX. Four resistance genes were successfully cloned into the pSWEET-Δ*bgaBcat* vector, making a total of seven genes cloned into this vector: *bla, cat, aph(3')-IIIa, ermB, mphA, vatD,* and *aph(2")-Ia* (*vatD, mphA,* and *aph(2")-Ia* were cloned by other members of the Wright lab).

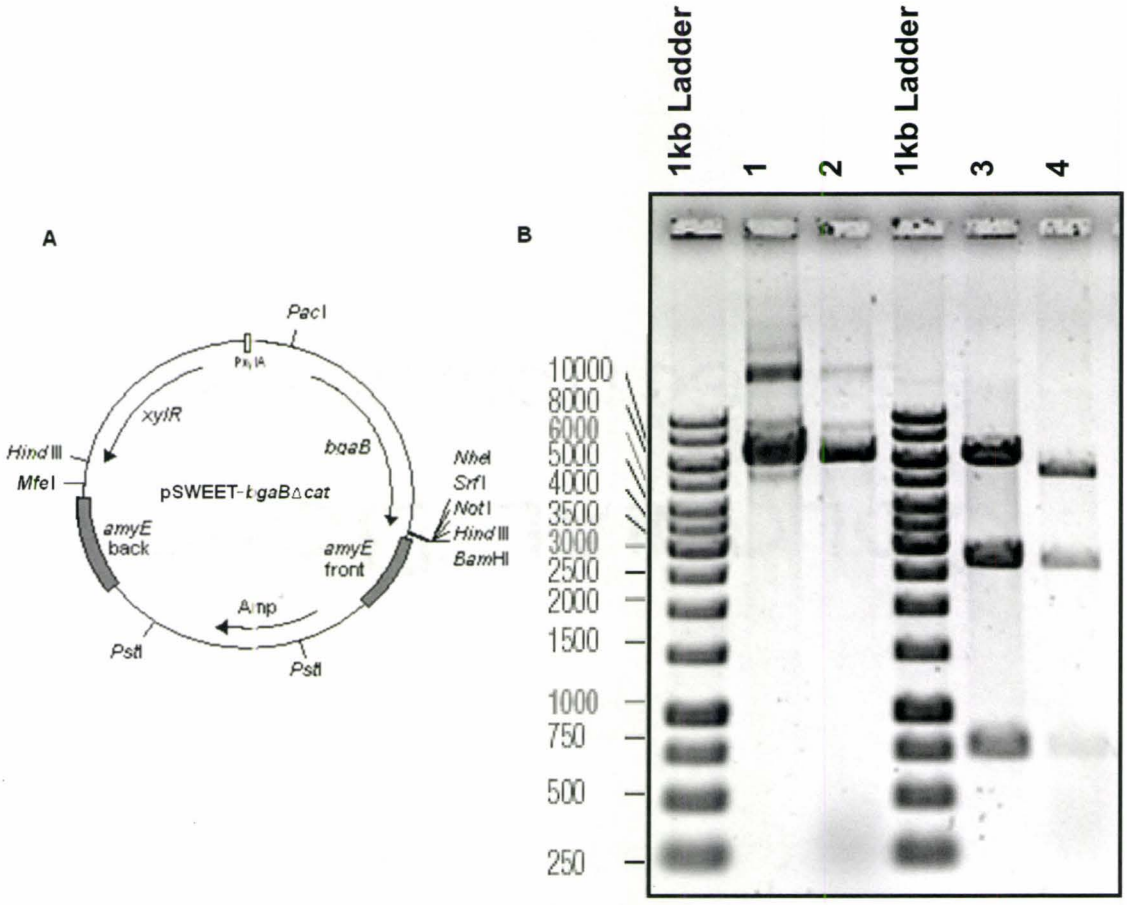


Figure 3.2. pSWEET-*bgaBΔcat*. **A)** Map of pSWEET-*bgaB*Δ*cat* clone. **B)** DNA agarose gel of a restriction enzyme digest of the pSWEET-*bgaB*Δ*cat* clone to verify *cat* gene deletion. Lanes: **1** pSWEET-*bgaB*, **2** pSWEET-*bgaB*Δ*cat* clone, **3** pSWEET-*bgaB* digested with *Hind*III, and **4** pSWEET-*bgaB*Δ*cat* clone digested with *Hind*III. Gene deletion is noted by a decrease in the size of the largest DNA fragment by 651bp when cut with *Hind*III. Gel was stained with 1x Sybr® Safe DNA stain.

3.2 – Integration of Antibiotic Resistance Genes into the *B. subtilis* Chromosome

With the success of cloning four resistance genes individually into pSWEET-Δ*bgaBcat*, the next step was to transform them into competent *B. subtilis* cells. Competent *B. subtilis* cells were successfully grown, however before transformations began, the working concentrations of each drug had to be determined. This was completed by streaking out the *B. subtilis* strain, BS1A1, on LB agar plates with different concentrations of drug, as well as setting up simulated transformations. Each simulated transformation consisted of $1/10^{th}$ of the normal plated volume of a *B. subtilis* transformation. The normal transformation volume of 201 µL -220 µL should consist of 100 µL of *B. subtilis* BS1A1 cells, grown to competency, 100 µL of SPII media (containing 4% xylose, for a final concentration of approximately 2% xylose), and anywhere from 1 µL -20 µL of linear DNA (21). In the simulated transformations, 20 µL of ddH_2O was added to the 200 µL mixture of cells and media instead of DNA. Of each simulated transformations, 20 µL was plated onto an LB/agar plate of a certain drug concentrations and 2% xylose, as well as being streaked out on a separate plates of the same drug concentration and 2% xylose. The plates were then incubated at 37°C for 18 hours and checked for colony formation. Results of the simulated transformations as well as controls are seen in table 3.

Antibiotic Concentration	Growth	
Erythromycin (µg/mL)	**Streak**	**Plate 20µL[a]**
0.5	-	-
1.0	-	-
2.0	-	-
5.0	-	-
Kanamycin (µg/mL)	**Streak**	**Plate 20µL[a]**
1.0	++	+
2.0	+	+
5.0	-	-
10.0	-	-
Ampicillin (µg/mL)	**Streak**	**Plate 20µL[a]**
0.5	+	-
1.0	-	-
2.0	-	-
5.0	-	-
Chloramphenicol (µg/mL)	**Streak**	**Plate 20µL[a]**
1.0	++	+++
2.0	+	-
5.0	-	-
10.0	-	-

Controls				
Plate	**Plated**	**Control type**	**Plasmid**	**Growth**
LB	none	negative	none	-
LB	20µL cells/SPII	positive	none	+++
LB	Streak	positive	none	++
LB Cm 5.0ug/mL	Streak	negative	none	-
LB Cm 5.0ug/mL	20uL cells/SPII	positive - transformation	1.0uL pS-b[b]	26cfu[c]
LB Cm 5.0ug/mL	200uL cells/SPII	positive - transformation	10.0uL pS-b[b]	39cfu[c]

+++ bacterial lawn (immeasurable colony growth)
++ greater than 100 colonies
\+ less than 100 colonies
\- no growth

[a] BS1A1 competent cells/SPII media
[b] pSWEET-*bgaB*
[c] colony forming units

Table 3. Antibiotic concentration test and transformation controls for *B. subtilis*. Plates containing different concentrations of four different antibiotics were either streaked with *B. subtilis* BS1A1 or were plated with 20µl of a mock transformation. All plates were LB agar and contained 2% xylose.

From the results of this experiment, an approximate MIC value was determined for each drug for the sole purpose of *B. subtilis* transformations. The following concentrations were used as the MIC value for each drug for *B. subtilis*: ampicillin 1μg/mL, erythromycin 0.5 μg/mL, kanamycin 5 μg/mL, and chloramphenicol 5 μg/mL.

Before transforming *B. subtilis*, all pSWEET constructs were digested with *Pst*I in order to linearize the vector, with the exception of pSWEET-*blaΔcat,* which was digested with *Apa*I and *Aat*II due to the presence of a *Pst*I site in the β-lactamase gene. It was important to use linear DNA since linearized DNA is taken up into the cells with greater efficiency than circular DNA in *B. subtilis* (21)

Transformations were performed using the same protocol as the simulated transformations, seen in table 3, however linear pSWEET DNA was used in the transformations. Most transformations resulted in 0-5 colonies per plate and these colonies were then subject to further testing to verify whether or not they were bonafide chromosomal *amyE* integrants.

3.3 – Verification of Integration into *B. subtilis* Chromosome

To ensure the authenticity of the transformation results, a series of controls were performed. To test for plasmid DNA contamination, the same transformation was also plated on LB agar plates supplemented with 5.0 μg/mL chloramphenicol and 2% xylose, which resulted in the growth of no colonies and was a successful negative control. A second negative control, to test for the presence of microbial contamination, was to plate

and streak with only H_2O on LB agar plates, this control also resulted in the growth of no colonies. To verify *B. subtilis* competent cell viability, a transformation with H_2O instead of plasmid DNA was performed and plated on LB agar plates with 2% xylose but that were not supplemented with an antibiotic. This positive growth control resulted in a bacterial lawn and confirmed cell viability. As a positive control for transformation efficiency, and to check for competency, *B. subtilis* competent cells were transformed with linear pSWEET-*bgaB*. These transformations resulted in 10-40 colonies when plated on LB agar plates supplemented with 5.0 µg/mL chloramphenicol and 2% xylose, with an average efficiency of 25 colonies per µg of linear DNA.

Transformations of competent *B. subtilis* cells with various linear pSWEET DNA constructs resulted in an average of 0-5 colonies per plate for the different drug concentrations. Further testing to verify whether or not they were chromosomal *amyE* integrants included screening on starch and PCR amplification of the chromosomal *amyE* region.

Transformants were assessed for chromosomal *amyE* disruption by counter-selecting on LB plates containing 1% starch, since *amyE* is an alpha-amylase gene and is required for starch metabolism. When grown on plates containing starch, the presence of a clear halo around the region of bacterial growth, after flooding with Gram's Iodine Solution (Sigma-Aldrich, St. Louis, MO), is indicative of starch utilization by an intact *amyE* gene and, therefore, a negative integration result (18). Therefore the absence of a halo indicates a disruption in the *amyE* gene, and a positive transformation and recombination result of the linear pSWEET DNA into the *B. subtilis* chromosome (see

figure 3.3) (18). All wildtype *B. subtilis* strains were able to utilize the starch in the LB 1% starch plates and therefore a halo was seen around all wildtype *B. subtilis* bacterial growth. The *B. subtilis amyE* disruption strain, EB648, was used as a negative control for starch utilization. EB648 is genotypically the same strain as the wildtype strain BS1A1, with the exception of a disruption at the *amyE* locus by the introduction of a *ydiB* gene (strain from unpublished research of E. D. Brown's lab, McMaster University).

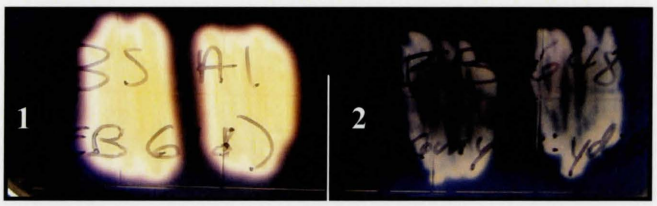


Figure 3.3. An LB agar 1% starch plate stained with Gram's Iodine Solution. Two *B. subtilis* strains were plated on 1% starch. Section **1** is wildtype BS1A1 cells, and shows a negative result for an *amyE* gene disruption. Section **2** is a modified strain of BS1A1 cells, EB648, which has an inactivated *amyE* gene. This strain was used as a negative control for starch utilization since no starch is utilized around the growth, which is seen by the absence of a clear halo when stained with Gram's Iodine Solution.

In addition to a phenotypic screen of selecting for positive transformants, by antibiotic selection and counter-selection for starch utilization, colonies were also genotypically screened using PCR techniques. This tested for the presence of a resistance gene in the chromosome, in the colonies that were resistant to drug, as well as to test for chromosomal disruption at the *amyE* locus.

To test for gene disruption by PCR, primers for the alpha-amylase gene, *amyE,* were used and when the PCR product was run on a 1% TAE agarose gel electrophoresis, a band was seen at approximately 1980bp, the expected size for *amyE* (see figure 3.4). This technique was then applied to the colonies that were growing on plates containing antibiotic. These colonies also displayed starch utilization when plated on 1% starch, however colony PCR was also employed to check for a disruption of *amyE.* Colony PCR reactions, using primers for *amyE,* were set up using colonies from the transformations of the pSWEET-*cat,* pSWEET-*ermB,* pSWEET-*aph(3')-IIIa,* and pSWEET-*bla* constructs. PCR products were run on a 1% TAE agarose gel and displayed DNA fragments that were approximately 1980bp in size, which indicated that the *amyE* genes were still intact and had not been disrupted by a double recombination event (see figure 3.5).

In addition to using primers for the *amyE* gene, primers that were complimentary to pSWEET, adjacent to the *amyE* fragments, were also constructed. The reverse primer was complimentary to a sequence between the *amyE*-back gene segment (see figure 3.1) and the *xylR* gene on pSWEET, and the forward primer was complimentary to a sequence between the multi-cloning site (MCS) and the *amyE*-front gene segment (see figure 3.1) on pSWEET. The reverse primer for *amyE* on pSWEET was then paired with the forward primer complimentary to the region upstream of *amyE* on the chromosome, and the forward primer for *amyE* on pSWEET was paired with the reverse primer complimentary to the region downstream of *amyE* on the chromosome (see table 2 for primers). Therefore, amplification of the *amyE* fragments would only occur if the resistance gene had recombined with the *B. subtilis* chromosome in the location of the *amyE* gene (see

figure 3.5). Unfortunately, there were no PCR products obtained from this experiment, with the exception of the positive control, a transformant from the pSWEET-*bgaB* transformation. In attempt to troubleshoot this problem, the PCR reaction was executed with primers of different lengths, different template concentrations, various dNTP concentrations, and multiple annealing temperatures. Since these efforts did not prove to be effective, and due to the inconsistency of the PCR results, colony PCR was no longer used for future experiments.

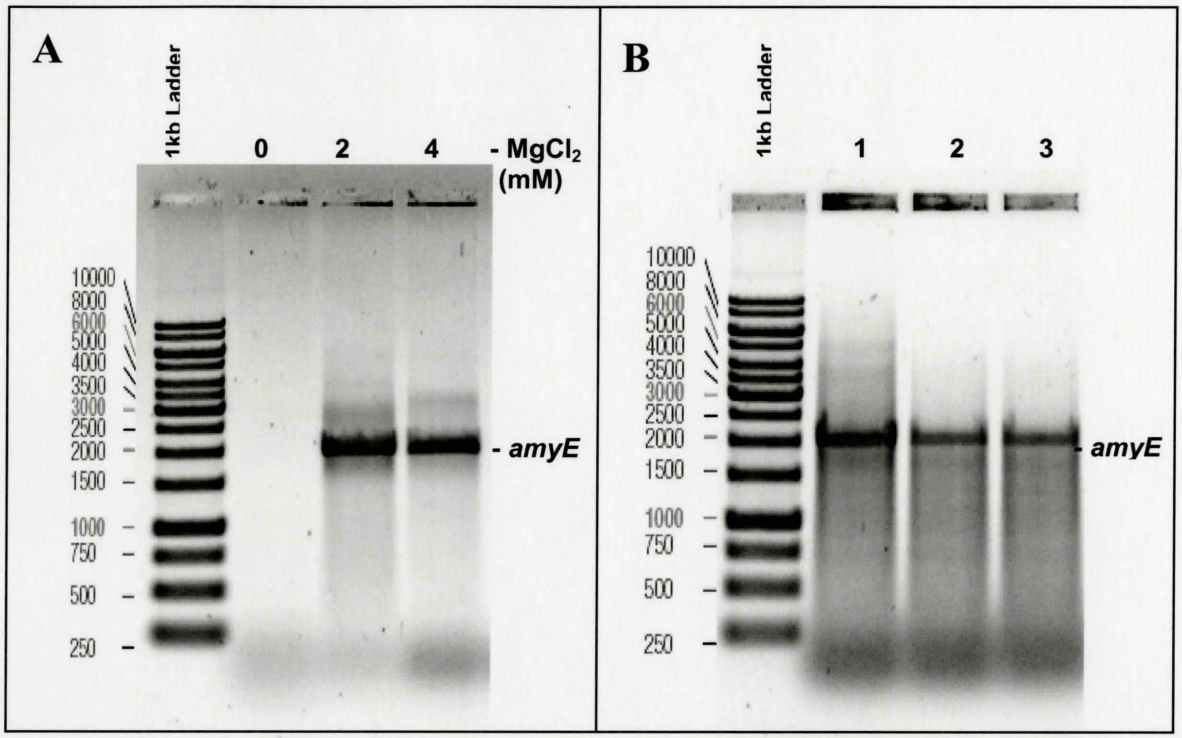


Figure 3.4. PCR amplification of the *amyE* gene. PCR products were run on 1% TAE agarose gels and primers for *amyE* were used to determine the condition of the 1980bp *amyE* gene. **A.** Optimization of the PCR reaction using *B. subtilis* BS1A1 cells and different concentrations of $MgCl_2$. **B.** PCR reaction using colonies from an *ermB* transformation as the template DNA, and 4mM $MgCl_2$. Lanes 1, 2, and 3 correspond to the colony number from the plated transformation. Both gels display the presence of intact *amyE* genes. Results for resistant colonies from transformations of the other genes were similar, if not identical.

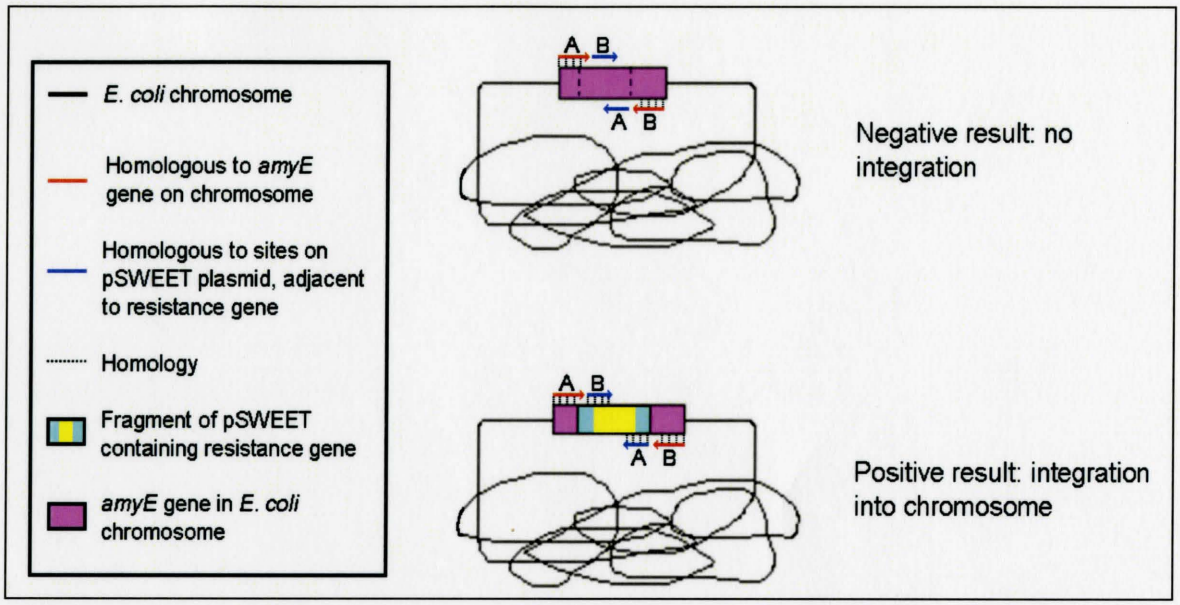


Figure 3.5. PCR amplification of *amyE* using two primer sets. This diagram shows that a negative result of no chromosomal integration would not result in any PCR amplification using primer set A or B since the primers homologous to sites on the pSWEET plasmid, adjacent to the resistance gene, would not have a site of homology to anneal to on the chromosome. Conversely a positive integration result would result in PCR amplification of the integrated site for both sets of primers, A and B, since all primers would have sites of homology to anneal to. Therefore, amplification of the *amyE* fragments with primer set A and primer B would only occur if the resistance gene had recombined with the *B. subtilis* chromosome in the location of the *amyE* gene.

In place of colony PCR techniques, genomic DNA was isolated with Instagene Matrix. Although this technique was less time-consuming, it did not prove effective due to poor DNA yield. This was also attributed to the thick peptidoglycan layer characteristic of Gram positive cells. Since the process of boiling the *B. subtilis* cells was not sufficient to fully lyse the cells, phenol-chloroform extraction was used to isolate genomic DNA for all further experiments.

After multiple failed attempts to acquire a double recombination event in the *B. subtilis* chromosome, each gene, as well as the vector, was studied. It was learned that the ribosome binding site (RBS) of pSWEET-*bgaB* was located between the *Nhe*I and *Pac*I restriction enzyme cut sites, therefore when *bgaB* was removed from the vector by digestion with *Nhe*I and *Pac*I, the RBS was also removed. Since the ribosome-binding site is a crucial component of a gene, it was decided to clone in the RBS for each pSWEET clone. This was accomplished by using PCR and Quickchange site directed mutagenesis protocols (see table 2 for primers). Initially, short Quickchange primers were used, however the regions homologous to the plasmid DNA were not long enough to properly anneal to the template DNA so primers with longer regions of homology were used, which proved to be successful. All *B. subtilis* transformations were carried out with the new clones containing a ribosome binding site for the resistance gene.

Transformations with the new clones were performed and plated on the respective drug and incubated. Resistant colonies were then patched onto LB agar plates with ampicillin (2 μg/mL) and 2% xylose to test for the possibility of single recombinants, and on LB agar plates with 1% starch to test for an *amyE* gene disruption caused by a double

recombination event. It would not be possible for a single cross-over event to occur using linear DNA, as the cells would not be viable, however a single-crossover could occur in the presence of an intact plasmid, due to an incomplete *PstI* digestion. Such an event would result in the inclusion of the ampicillin resistance cassette in pSWEET and an ampicillin resistant phenotype. All resistant colonies initially grew on ampicillin and also continued to utilize starch. Due to the low probability of a double recombination event occurring after only plating the cells once, colonies that were resistant to the respective drug were streaked out to obtain isolated colonies. These new colonies were then patched onto grids on LB agar plates with ampicillin (2μg/mL) and 2% xylose, LB plates with the respective drug, as well as LB agar plates with 1% starch. This cycle of streaking and patching was carried out for more than 10 plating cycles. Eventually colonies grew which were resistant to the respective antibiotic and sensitive to ampicillin, specifically the pSWEET-*aph(3')-IIIaΔcat* transformants which were kanamycin resistant and ampicillin sensitive, however these clones continued to utilize starch which would infer an intact *amyE* gene.

To verify the presence of an intact *amyE* gene, as well as the presence of the *aph(3')-IIIa* gene, PCR was employed. For this experiment, colonies were grown in the presence of kanamycin (5 μg/mL) and 2% xylose, and incubated overnight at 37°C. Genomic DNA was then isolated using phenol-chloroform extraction and was used directly in the PCR reaction. Primers specific for the *amyE* gene in the chromosome were used, as well as primers specific for the *aph(3')-IIIa* gene (see table 2 for primers) and PCR products were separated by DNA gel electrophoresis. In figure 3.6, the four clones

that were examined displayed DNA fragments of 1980bp when the *amyE* primers were used, and 795bp DNA fragments when the *aph(3')-IIIa* primers were used. As expected, the two colonies from control strains, BS1A1, the wildtype *B. subtilis* strain, and EB648, the *amyE* disruption strain, had no amplified DNA fragments from the primers specific for *aph(3')-IIIa*. As well, a 1980bp fragment was amplified from the BS1A1 DNA, but not from the EB648 DNA, with primers specific for the *amyE* gene (see figure 3.6). Since these four clones were resistant to kanamycin (5µg/mL), sensitive to ampicillin (2µg/mL), and still utilized starch, it is possible that the kanamycin resistance gene, *aph(3')-IIIa,* recombined elsewhere in the chromosome. As well, these clones only grew on LB agar plates with kanamycin (5µg/mL) if the plates contained 2% xylose; they did not grow on LB agar plates with kanamycin (5µg/mL) that did not contain xylose. This suggests that *aph(3')-IIIa* was still under the control of the xylose system, but likely elsewhere in the *B. subtilis* chromosome. As well, using PCR techniques, it was confirmed that the each of the four clones tested had intact *amyE* genes but also had the *aph(3')-IIIa* gene somewhere in the chromosomes. Due to these difficulties, it was decided that the focus of the project would switch to cloning resistance genes into Gram negative bacteria and using an expression system suitable for *E. coli* cells to screen for inhibitors.

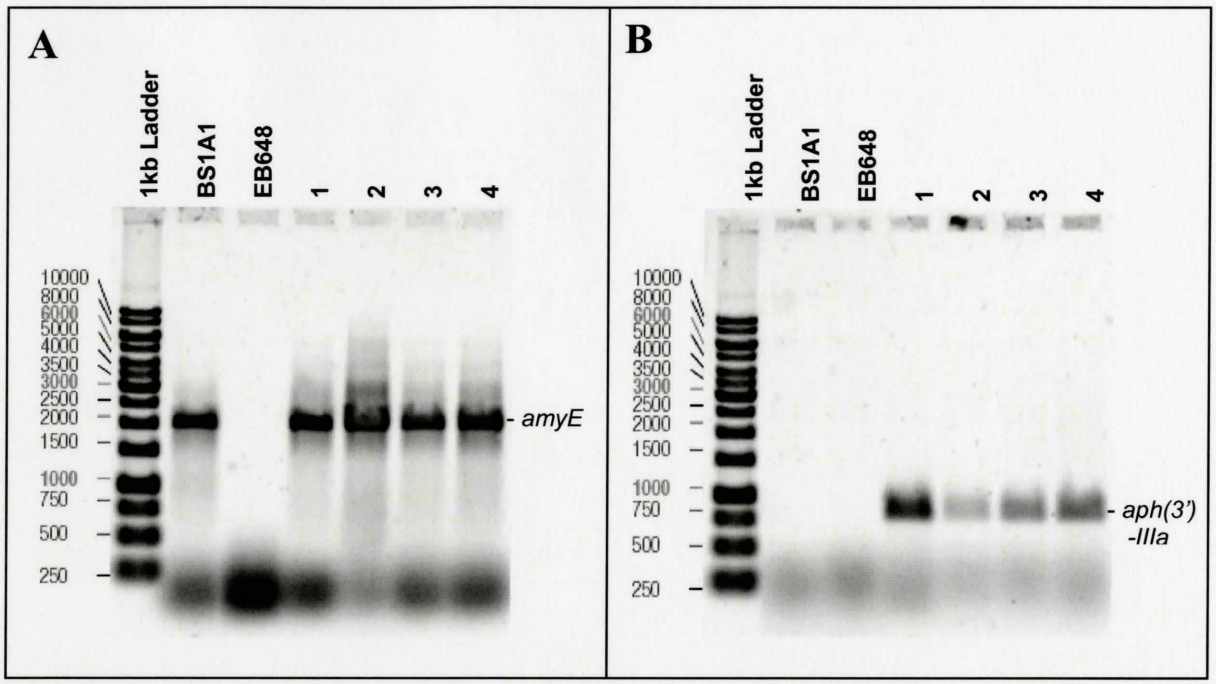


Figure 3.6. Detection of the *amyE* and *aph(3')-IIIa* genes using PCR. PCR products were separated on 1% TAE agarose gels and stained with Sybr Safe DNA stain (Invitrogen, Burlington, ON). Lane numbers 1, 2, and 3 correspond to the number of the colony that was isolated from the plate. **(A)** Primers specific for the chromosomal *amyE* gene in *B. subtilis* were used to detect the presence of an intact alpha-amylase gene in the chromosome. **(B)** Primers specific for the *aph(3')-IIIa* gene were used to detect the presence of the kanamycin resistance gene, aminoglycoside phosphotransferase type IIIa, in the *B. subtilis* chromosome.

3.4 – The pETcoco Expression System

The pETcoco expression system (Novagen) is a suitable vector to express resistance genes in Gram negative bacteria, namely *E. coli.*, since it allows for the vector copy number to be manipulated, from single copy to 40 copies per cell (35). It has a T7 promoter which yields good expression of the gene of interest and can be induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) when in a host expressing the T7 RNA polymerase (35). This vector contains replication elements of a single-copy genomic cloning vector as well as a medium-copy plasmid: The genes *oriS, repE,* and *parABC* control the single-copy state and are induced when grown in LB with 0.2% glucose, and the genes *trfA, araC-*P_{BAD} and *oriV* control the medium-copy state and are induced when grown in LB with 0.01% arabinose (35). pETcoco-1 also contains cloning elements such as the T7 promoter, T7 terminator, *lacI* gene, and a chloramphenicol resistance gene for selection (35). The pETcoco-1 system is identical to the pETcoco-2 system, however pETcoco-2 has an ampicillin resistance gene in place of the chloramphenicol resistance gene present in pETcoco-2 (see figure 3.7) (35). Therefore pETcoco-2 may be used in future experiments when the gene to be expressed in the system is the chloramphenicol acetyltransferase.

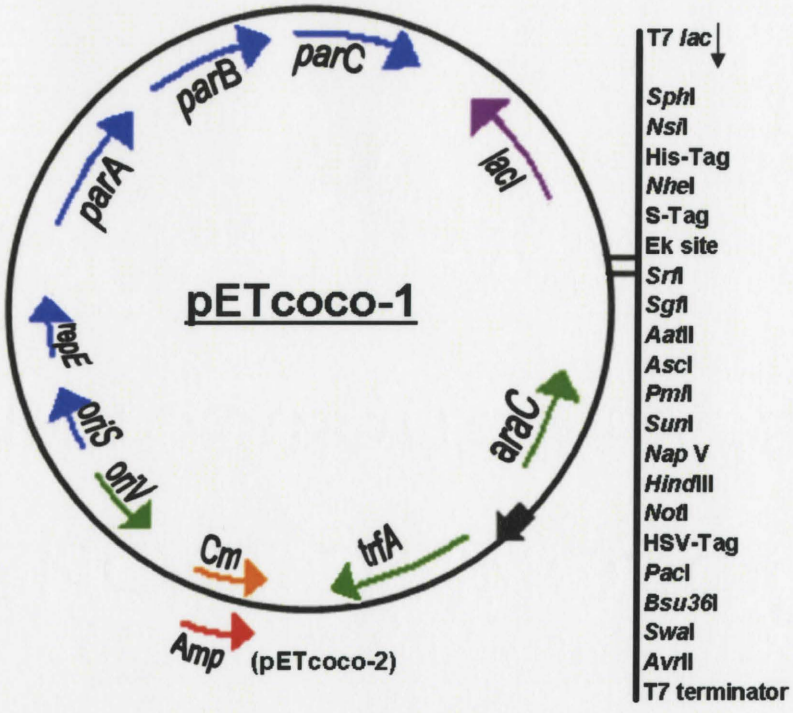


Figure 3.7. Vectors pETcoco-1 and pETcoco-2. The single copy state is controlled by genes *repE, oriS,* and *parABC* and is induced in the presence of 0.2% glucose. The medium copy state is controlled by genes *trfA, araC,* and *oriV,* and is induced in the presence of 0.01% arabinose. The single difference between the two plasmids is the resistance gene: pETcoco-1 has a Cam^R gene and pETcoco-2 has an Amp^R gene. (Adapted from Sektas 2003)

3.5 – Transformation into Tuner cells and Confirmation of Copy-Control

The pETcoco-1 vector was purchased from Novagen (Gibbstown, NJ) and transformed into *E. coli* Top10 cells. The transformation was plated on LB agar plates supplemented with 12.5 μg/mL chloramphenicol and incubated at 37°C overnight. As well, in order to ensure the efficacy of the copy-number control in pETcoco-1, the transformation of *E. coli* Top10 cells with pETcoco-1 was also plated on three types of plates: LB agar with Cm 12.5μg/mL and 0.2% glucose, LB agar with Cm 12.5 μg/mL, and LB agar with Cm 12.5 μg/mL and 0.01% arabinose. These were incubated overnight at 37°C and then three colonies were picked from each plate and each used to inoculate three liquid cultures supplemented with different carbohydrates: LB with Cm 12.5 μg/mL and 0.2% glucose, LB with Cm 12.5 μg/mL, and LB with Cm 12.5 μg/mL and 0.01% arabinose. The nine cultures were incubated overnight at 37°C, then the plasmid DNA was isolated and digested with *Bam*HI for 2 hours at 37°C in order to linearize the vector. The digests were then separated by DNA gel electrophoresis (see figure 3.8). The intensity of the bands of the digested DNA indicates that the copy number of the plasmid varies from the basal level when the plasmid replicates in the presence of glucose or arabinose; it is lower in the presence of 0.2% glucose, and higher in the presence of 0.01% arabinose. However it is not affected by whether there is glucose or arabinose in the original plate used to inoculate a culture for a plasmid prep.

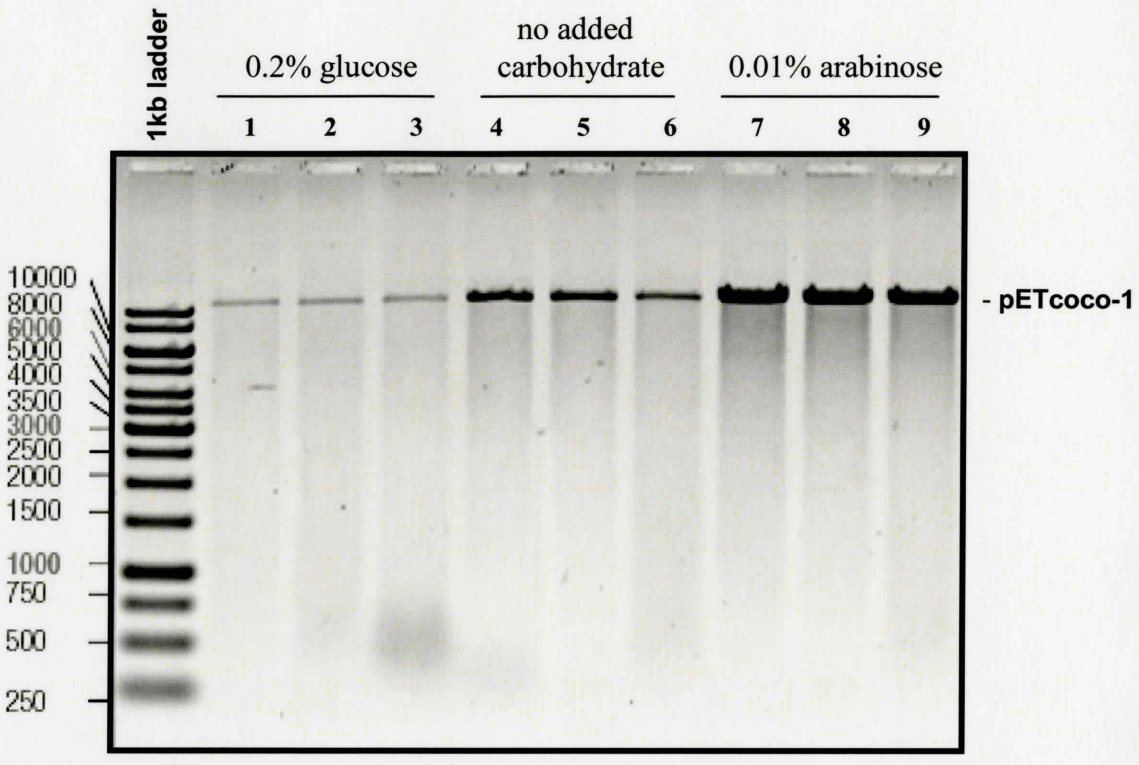


Figure 3.8. Verification of the copy control of pETcoco. Bam HI digests of the pETcoco-1 plasmid isolated from cultures grown in media containing different sugars. **1-3** were grown in LB with 0.2% glucose, **4-6** were grown in LB with no added sugar, and **7-9** were grown in LB with 0.01% arabinose. The cultures that the plasmids were prepped from were first inoculated with colonies from different types of plates; **1**, **4** and **7** were from LB agar plates with 0.2% glucose, **2**, **5**, and **8** were from LB agar plates with no added sugar, and **3**, **6** and **9** were from LB plates with 0.01% arabinose. All plates and cultures were supplemented with 12.5µg/mL chloramphenicol.

3.6 – Amplification of Resistance Genes for pETcoco and Vector Construction

The resistance genes *aph(3')-IIIa* and *bla* were PCR amplified using oligonucleotides which contained restriction digest sites for *Nhe*I and *Hind*III (see table 2 for primers). The previously constructed pSWEET clones, pSWEET-*aph(3')-IIIaΔcat* and pSWEET-*blaΔcat,* were used as the template DNA in the PCR reactions. The PCR reactions were separated by DNA gel electrophoresis, gel extracted using the QIAEX II kit and digested with *Nhe*I and *Hind*III for 2 hours at 37°C. The digested PCR products were then again gel purified and extracted. The pETcoco-1 vector, which was previously transformed into *E. coli* Top10 cells, was isolated and digested with *Nhe*I and *Hind*III for 2 hours at 37°C, and gel purified and extracted. The PCR-amplified genes and the pETcoco-1 vector were then used in ligation reactions to construct the new vectors. Difficulties ligating were approached by altering DNA concentrations and the ratios of vector to insert, using different quantities of T4 DNA ligase, using newly purchased ligase and ligase buffer, making new batches of electrocompetent *E. coli* Top10 cells, using different gel-extraction methods, de-salting the ligation mix using the QIAEX II DNA desalting protocol before transformation via electroporation, and using different electrocuvette sizes. The ligation reactions were transformed into *E. coli* Top10 cells and transformants were selected on LB agar plates supplemented with 12.5 μg/ml chloramphenicol. Successful pETcoco-*bla* and pETcoco-*aph(3')-IIIa* ligation reactions were confirmed by restriction mapping, as well as by sequencing at MOBIX using a pETcoco-1 sequencing primer (see table 2).

3.7 – Transformation into *E. coli* Tuner cells and Induction of pETcoco-*bla*

The pETcoco1-*bla* and pETcoco-*aph(3')-IIIa* clones were transformed into *E. coli* Tuner cells (Novagen, Gibbstown, NJ) by electroporation. Tuner cells were selected since they are BL21(DE3) cells, which contain a *lacZY* deletion mutation. The lac permease mutation (*lacY*) allows for uniform entry of IPTG into the cells when inducing for protein expression of the cloned gene (Novagen).

For screening purposes, the β-lactamase gene, *bla,* would already have a positive control for an inhibitor, clavulanic acid. Therefore it was decided that the pETcoco-*bla* construct would be studied and optimized first. To test whether the *bla* gene was properly cloned in and active, Tuner cells which were transformed with pETcoco1-*bla* were streaked out on LB agar plates supplemented with Cm 12.5 μg/mL, as well as LB agar plates supplemented with 50 μg/mL ampicillin and coated with 40 μL IPTG (100mM), and LB agar plates with 50 μg/mL ampicillin and no IPTG. Plates were incubated overnight at 37°C. Growth occurred on all plates except for plates that only contained 50 μg/mL ampicillin and no IPTG. Therefore this result indicated that the *bla* gene was active and under the control of the T7 promoter.

3.8 – Antibiotic Minimal Inhibitory Concentrations

Minimum inhibitory concentrations (MIC) were determined for *E. coli* Tuner cells containing the pETcoco-*bla* vector. MICs for ampicillin were determined in the presence of different concentrations of IPTG in a 96 well plate format, as a checkerboard assay (see figure 3.10). In order to induce the *bla* resistance gene under the control of the T7 promoter the following concentrations of IPTG were used: 0mM, 0.016mM, 0.032mM, 0.062mM, 0.125mM, 0.250mM, 0.50mM, and 1.0mM. A uniform concentration of 5μg/mL chloramphenicol is added to all wells, with the exception of the sterility controls, in order to maintain the pETcoco1-*bla* vector in the cell. As well, the MICs of ampicillin, in the presence of different IPTG concentrations, were performed in the presence of 0.2% glucose, no added carbohydrate, and 0.01% arabinose. These results suggest that the presence of glucose, which reduces the plasmid copy-number, and the presence of arabinose, which increases the plasmid copy number, do not significantly affect the MIC of ampicillin. However, the MIC for ampicillin changes directly with the amount of IPTG that is used, in that the greater the concentration of IPTG, the higher the MIC range. Therefore the level of gene expression is directly regulated by IPTG and is not influenced by the number of copies of the plasmid in the cell. This comparison of results can be seen in table 4.

Since clavulanic acid was to be used as a positive control it was necessary to find out the MIC to determine the amount of clavulanic acid needed to inhibit the *bla* gene in screening conditions. As well, it was used to validate a change in the level of gene expression of *bla* when induced with different concentrations of IPTG. The MIC assay

was performed in a 96 well plate without IPTG, and with 0.031 μg/mL of IPTG, in the presence of three different ampicillin concentrations: 0 μg/mL, 4 μg/mL, and 64 μg/mL. The results from these MIC assays can be seen in table 5 and show that the MIC of clavulanic acid is 32 μg/mL in the absence of ampicillin, and 0.125 μg/mL in the presence of 4 μg/mL of ampicillin. It also shows that by upregulating the level of *bla,* by inducing with IPTG, the MIC of clavulanic acid increases, resulting in an increased level of β-lactamase in the cell. These β-lactamase molecules then need to be inactivated by clavulanic acid in order for ampicillin to successfully kill the cells.

From the results of these assays, it was decided that the following concentrations would be used for screening: 0 mM IPTG in order to simplify the assay, 4 μg/mL ampicillin as the sub-inhibitory concentration, and 0.125 μg/mL clavulanic acid as a positive control for inhibition.

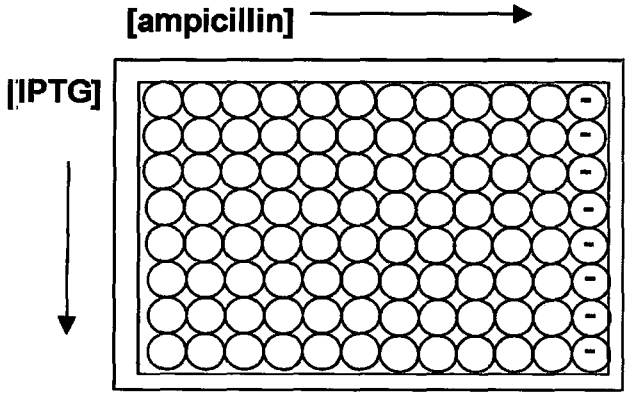


Figure 3.9. Design of ampicillin MIC with IPTG. Design to of an MIC for ampicillin, against IPTG concentration, arranged as a checkerboard assay. The last column of wells on the right only contained media, without antibiotics or inoculum, to serve as a sterility control.

IPTG (mM)	MIC of ampicillin (µg/mL) for *E .coli* pETcoco-*bla*		
	Glucose	**No Carbohydrate**	**Arabinose**
0	8	8	16
0.016	64	64	64
0.031	128	256	256
0.062	512	512	512
0.125	>1024	>1024	>1024
0.25	>1024	>1024	1024
0.5	512	512	1024
1.0	512	512	512

Table 4. Ampicillin MICs with different IPTG concentrations Ampicillin MICs in the presence of different concentrations of IPTG. The MICs for the eight IPTG concentrations were also tested in the presence of glucose, and arabinose, in addition to the standard media without an added carbohydrate. As well all media was supplemented with 5 µg/mL of chloramphenicol to maintain the pETcoco-*bla* plasmid.

Ampicillin concentration (μg/mL)	**MICs of clavulanic acid (μg/mL) for *E. coli* pETcoco-*bla***	
	0mM IPTG	0.031mM IPTG
0	32	32
4	0.125	0.5
64	0	0.125

Table 5. Clavulanic acid MICs. MICs of clavulanic acid for *E. coli* pETcoco-*bla* in the absence of IPTG and with 0.031 mM IPTG, in the presence of 0 μg/mL, 4 μg/mL, and 64 μg/mL of ampicillin.

3.9 – Screen optimization

To optimize for screening, the patterns of growth, along with growth in different concentrations of DMSO, and growth from different dilutions of inoculum, had to be assessed. All growth curves were performed for a 26 hour period in the presence of 4 μg/mL of ampicillin. Growth curves were completed with *E. coli* Tuner cells, *E. coli* Tuner cells containing pETcoco, and *E. coli* Tuner cells containing pETcoco-*bla*. Results of these growth curves can be seen in figures 3.10.

A growth curve for *E. coli* Tuner cells containing pET-coco-*bla* was completed using different inoculum concentrations to test for changes in the rate of cell growth. The following inoculum dilutions were used: 1/1, 1/10, 1/100, 1/1000, 1/10000, and 1/100000. Results of this growth curve can be seen in figure 3.11. It appeared that dilutions of 1/1, 1/10, and 1/100 would be sufficient to begin proper growth however dilutions of 1/1000 and 1/10000 would not be sufficient to begin proper growth.

A growth curve for *E. coli* Tuner cells containing pET-coco-*bla* was completed in the presence of DMSO to test for changes in cell growth since compound libraries are suspended in DMSO. The following concentrations of DMSO were used: 0%, 0.5%, 1.0%, 2.5% and 5%. Results of this growth curve can be seen in figure 3.12. It appeared that a concentration of 0%, 0.5% or 1% would have little effect on the growth of these cells, and a concentration of 2.5% would only have a minor effect. However a concentration of 5% significantly decreases the cell viability.

In following attempts to reproduce these results it was found that the MIC of ampicillin varied from 2 μg/mL to greater than 2000 μg/mL when the MIC assays were

performed multiple times. Upon reproduction of this assay, chloramphenicol was omitted since it was deemed unnecessary to maintain the plasmid inside the *E. coli* Tuner cells since there was an added selective pressure of ampicillin in the assays, which would encourage the production of the pETcoco-*bla* plasmid in these conditions. However, similar MIC results were found to be unattainable after multiple attempts. It was considered that perhaps these results conflicted with the original MIC results due to the lack of chloramphenicol in the media. Conversely, upon introducing 5 μg/mL chloramphenicol back into the assay the MIC value of ampicillin for pETcoco-*bla* was 2 μg/mL, which again unexpectedly varied from the original MIC value of ampicillin, for pETcoco-*bla* in the presence of 5 μg/mL, which was 8 μg/mL. These variations may be caused by plasmid instabilities which would need to be further investigated however due to time constraints these issues were not explored.

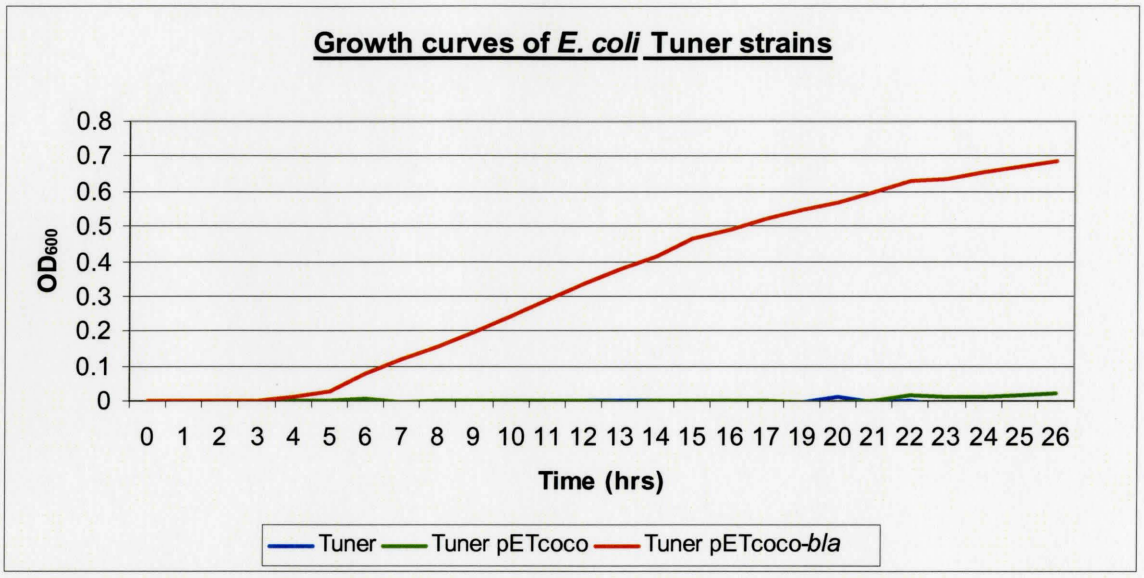


Figure 3.10. Growth curve of *E. coli* Tuner strains. Growth curves completed in the presence of 4 μg/mL of ampicillin over a 26 hour period. No growth was seen for the Tuner and Tuner pETcoco strains since there was no *bla* gene present to confer resistance.

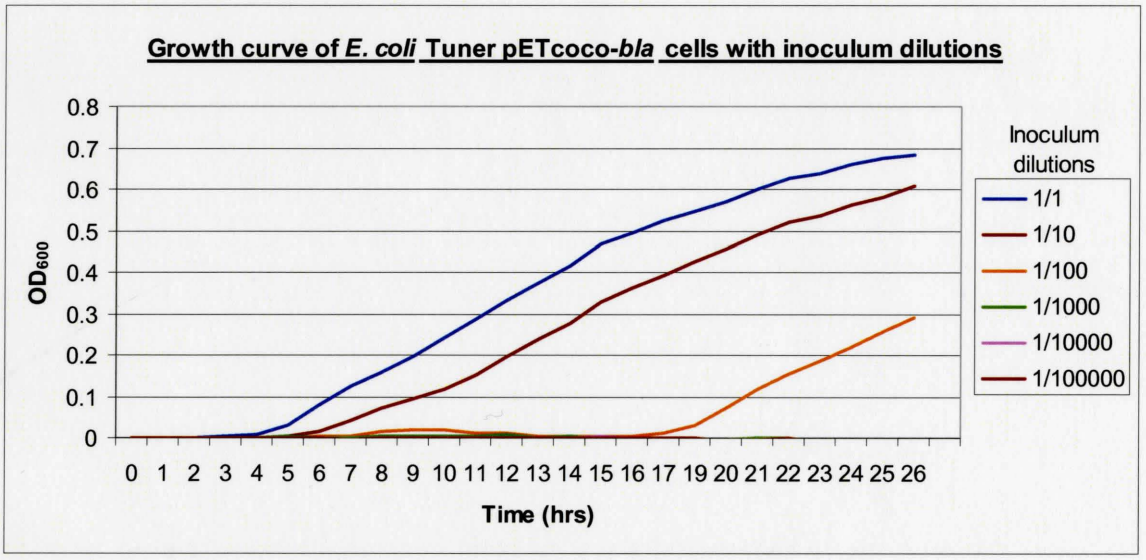


Figure 3.11. Growth curve of *E. coli* Tuner cells with pETcoco-*bla* with various inoculum dilutions. Growth curve completed in the presence of 4 μg/mL of ampicillin over a 26 hour period.

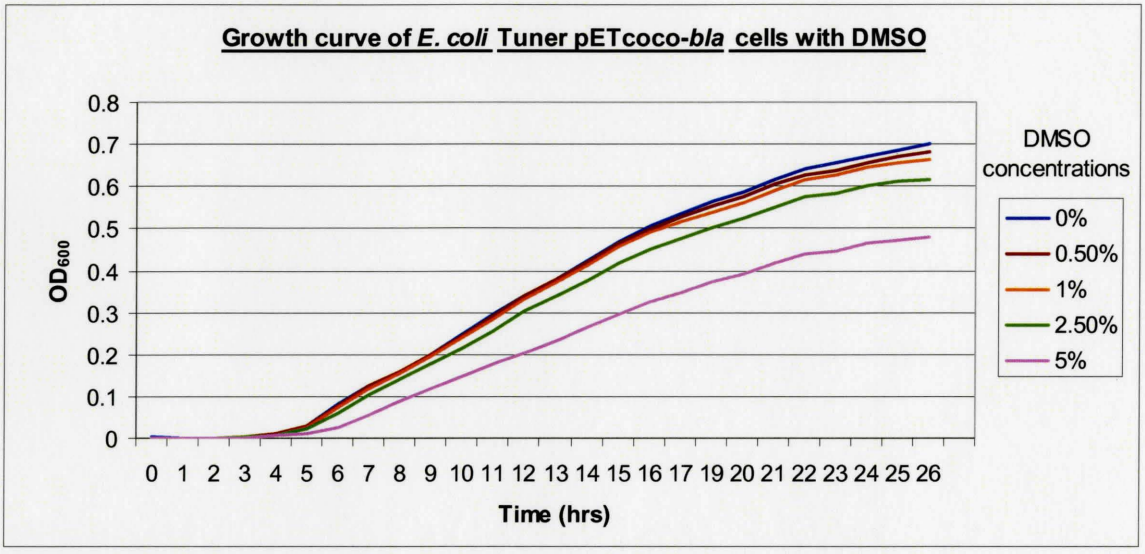


Figure 3.12. Growth curve of *E. coli* Tuner cells with pETcoco-*bla* with various DMSO concentrations. Growth curve completed in the presence of 4 µg/mL of ampicillin over a 26 hour period.

Chapter 4 – Discussion and Concluding Remarks

4.0 – Cell-based screen development

In order to combat the immense problem of resistant bacteria, there have been efforts to develop novel antibiotics and to modify existing antibiotics, however these approaches are beginning to reach the end of their limits. A third approach, searching for inhibitors of resistance mechanisms, which are the root of the problem, has not yet been fully explored. This may be due to the fact that searching for inhibitors, especially in the natural environment, is more complicated and less straightforward than searching for undiscovered antimicrobials. The ideal and desired inhibitory compound is one that can weaken, block, or inactivate the activity of a resistance enzyme so much that the antibiotic can be restored of its activity and eradicate nearby microorganisms. So therein lies the problems, how can one test for increased antimicrobial activity in the presence of a resistance mechanism, along with increased inhibitor activity, when the identity of the antimicrobial and the inhibitor are unknown? A resolution would be to start with a known antibiotic, preferably one such as ampicillin that can represent the β-lactam group of antibiotics which are still widely used and well characterized but are targets of resistance, and secondly to select a resistance enzyme which inactivates ampicillin, such as TEM β-lactamase. Then by developing an assay which could screen possible inhibitors in the presence of a sub-inhibitory concentration of ampicillin, using a strain of bacteria producing a consistent level of the β-lactamase enzyme, one could study the change in β-lactamase activity on ampicillin as a function of the cell viability in respect to the present inhibitory compound. TEM β-lactamase is the ideal enzyme to begin with as it has known inhibitors, clavulanic acid, sulbactam, and tazobactam, which could be used as positive

controls to test the validity of the screen. As well, this screen would also hold utility in dereplication studies. Dereplication has significant relevance in drug discovery due to the great challenges in discovering new antibiotics from environmental sources. The problem is that the majority of antimicrobials that are found to be produced by environmental, soil-dwelling bacteria, one of the best sources for chemotherapeutic agents, are ones that have preciously been discovered. For example, Richard Baltz has recalled early antibiotic discovery work by the pharmaceutical company Merck: Merck noted that if 10,000 actinomycetes were screened, approximately 2500 would produce an antimicrobial compound. However, 2250 of those would be streptothricin, 125 would be streptomycin, and 40 would be tetracycline. In the end, only having about 10 compounds holding potential as a new antibiotic, but most being ruled out due to high levels of toxicity (4). Merck was able to rule out these major groups of known antibiotics by counter-selection on antibiotic resistant hosts, a dereplication assay (4). Therefore by screening with bacterial strains expressing various resistance genes, novel antibiotics could be separated from those which are already known.

The development of this screen was the project goal, to be achieved through the use of the pSWEET expression system for the Gram positive bacterium *B. subtilis*, and the pETcoco expression system for the Gram negative bacterium *E. coli.* Due to the lack of a lipopolysaccharide outer membrane in Gram positive bacteria, a greater number of hits would be expected in the screening process, thus making Gram positive bacteria the first choice for screening purposes. However it was decided that expression systems for both types of bacteria would be developed as many pathogenic bacteria are Gram

negative, such as *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa,* and there is a great need for inhibitors which are bioactive against Gram negative species, those able to permeate their outer membrane. Therefore it would be advantageous to screen both groups of bacteria even though the number of hits obtained using Gram negative bacteria would be significantly less than that obtained using Gram positive bacteria.

To design a cell-based screen for inhibitors of resistance enzymes, expression systems must be cautiously chosen to ensure that they satisfy necessary qualifications. An optimum expression system for a screen of this type would possess qualities such as controlled expression levels, an inducible promoter, a multi-cloning site, the option of single-copy expression, tight regulation, minimal resistance cassettes, and reliable reproducibility.

The pSWEET expression system met many of the previously listed criteria for an adequate expression system. It is tightly regulated, is induced upon introduction of xylose into the system, only contains one chloramphenicol resistance cassette which was removed without difficulty, and allows for single-copy expression of the gene of interest, upon integration into the *B. subtilis* chromosome through a double recombination event at the *amyE* gene. This plan of integration proved to be the weakness of this system in respect to this project. As seen in figures 3.4 and 3.6, attempts to introduce a resistance gene, *aph(3')-IIIa* in this case, into the chromosome did not result in the expected phenotype of kanamycin resistant *B. subtilis* colonies which were unable to utilize starch due to a disruption at the alpha-amylase gene, *amyE.* Instead the colonies observed were resistant to kanamycin and capable of utilizing starch, which would infer an introduction

of the *aph(3')-IIIa* resistance gene into the chromosome and an intact *amyE* gene. In theory these bacteria would have needed to acquire the *aph(3')-IIIa* resistance gene elsewhere in the chromosome, and also have an intact alpha- amylase gene, in order to be kanamycin-resistant and able to utilize starch.

The occurrence of a single recombination event could produce a kanamycin-resistant, starch utilizing phenotype since this event would result in the integration of the *aph(3')-IIIa* gene without full disruption of the *amyE* gene. Although this single cross-over phenotype would not be possible when transforming with linear DNA, as the cells would not be viable, it could occur if the pSWEET-*aph(3')-IIIa* plasmid was still intact, perhaps due to an incomplete *Pst*I restriction enzyme digest. This would lead to an ampicillin-resistant phenotype since an uncut pSWEET-*aph(3')-IIIa* plasmid would still yield a β-lactamase gene. With that said, since the four clones seen in figure 3.6 were ampicillin-sensitive, it is likely that a double recombination event occurred and that the kanamycin-resistant phenotype was not caused by a single cross-over event. Therefore, since the four clones seen in figure 3.6 were resistant to kanamycin (5 μg/mL), sensitive to ampicillin (2 μg/mL), and still utilized starch, and since PCR results support the presence of intact *amyE* genes, it can be rationalized that the kanamycin resistance gene, *aph(3')-IIIa* may have recombined elsewhere in the chromosome. In order to investigate the location of this integration, backward primers homologous to the resistance gene, which would sequence away from the resistance gene, would be used. By sequencing the regions flanking the resistance gene at the sight of insertion, we would gain insight into the location of the resistance gene in the chromosome. As well, in order to determine the

location of the resistance gene, the *B. subtilis* chromosome could be digested with restriction enzymes and probed via Southern blot hybridization. Probes specific for the resistance gene of interest would be employed and would hybridize to the DNA fragments containing the resistance gene, which could then be sequenced and studied to locate the site of integration. However, due to these difficulties it was decided that for the remainder of the project the focus would be cloning resistance genes into the pETcoco expression system for Gram negative bacteria.

The pETcoco expression system for *E. coli* from Novagen also held potential for an adequate system to screen for inhibitors. It is tightly regulated by the T7-*lac* regulatory system, allowing for control of gene expression levels through the use of IPTG, it only contains one resistance cassette, and the plasmid can be maintained at the single-copy level by supplementing with glucose. However the weakness of this system in respect to this project was the unattainable consistency of results. Once the *bla* gene was cloned into the pETcoco vector, MIC levels for ampicillin, as well as the ideal concentration of IPTG for gene expression, were determined. The initial results held potential as they presented a system in which the level of gene expression could be easily manipulated as it appeared to be directly related to the concentration of IPTG added to the system, as seen in table 4, which was to be expected. More importantly, it was discovered that the plasmid copy number does not significantly affect the MIC value and additionally, without IPTG induction there was enough gene expression to confer resistance. This allows for a simplified screen without introduction of IPTG or an added carbohydrate. However, during the optimization stage, attempts to reproduce these results

reported inconsistent MIC values. The inability to reproduce a value such as the minimum-inhibitory concentration of ampicillin deemed the pETcoco system unworthy as an expression-system for the proposed screen at this point in time. However with further exploration of the cause of this variation in results may find that the pETcoco system is indeed the system of choice for cell-based screening in Gram negative bacteria. This wide range of results for the ampicillin MIC assay could be due to plasmid instability. The presence of the *bla* gene, at a low level of expression, may not have been sufficient to maintain the presence of the plasmid in the cell. Additionally, it may have been due to an event described as 'transitory plasmid instability', as discussed in the 1994 paper by Lamotte, *et al* (24). They found that ampicillin resistance can vary as a factor of the plasmid copy number in the culture, and thus cell growth, as a significant raise in copy number can infer a greater concentration of β-lactamase enzymes, encouraging a faster rate of ampicillin degradation (24). As well, since low concentrations of ampicillin can be degraded faster than higher concentrations of ampicillin by a fixed amount of β-lactamase enzymes, the time period of which a culture is incubated is directly related to the remaining concentration of ampicillin in the media and must be considered when growing such culture. Once all of the ampicillin in the culture has been broken down by β-lactamase, the culture is then subject to contamination by other microorganisms in the surrounding environment unless there is another antibiotic present in the media. Therefore, further testing to investigate the problem of plasmid instability may include optimization of growth conditions such as inoculum dilution, incubation time, and antibiotic concentration.

4.1 Future prospects

Although the pSWEET and pETcoco expression systems exhibited undesirable characteristics such as indeterminate integration, and poor reproducibility, respectively, these systems should not be completely discarded. The pSWEET system may show promise in the future if perhaps paired with a different strain of *B. subtilis*. In the work of Bhavsar 2001, the *B. subtilis* strains used for integration contained *amyE* genes which were previously inactivated by a resistance cassette of either a chloramphenicol acetyltransferase or an erythromycin resistant methylase, and were used to obtain successful integration results in their work. These genotypes were not used in this project to avoid the introduction of more than one resistance cassette into the microorganism. Therefore in future trials it would be beneficial to try to integrate the pSWEET fragment into these strains to determine if a double cross-over event would occur at the *amyE* locus if it was previously disrupted. As well, upon determining the current location of integration of the resistance genes into the *B. subtilis* chromosome, if the location had significant homology to the *amyE* locus, a strain with a deletion in this region could be used to avoid future integration at this site. Alternatively, if the location of integration does not affect the screen, or the fitness of the strain, the strain could then be successfully used in the proposed screen. In addition, the pETcoco expression system should not be abandoned completely. The initial MIC results indicated that the system could deliver the desired modification of the level of gene expression, however the stability of the plasmid in the cell, and the reproducibility of the level of gene expression, would have to be further studied, as mentioned in the previous section.

In the future, if these pSWEET and pETcoco expression systems had been successfully developed, the growth curves, inoculum dilutions, DMSO concentrations, MIC assays, xylose concentrations for pSWEET, and IPTG concentrations for pETcoco, would all have to be completed in order to be ready to screen a compound library. As well, for screens using natural product isolates grown in Medium A such as the growing library of over 600 bacterial natural product isolates in the Wright Lab, the same assays would have to be completed to optimize the screen once again, with the exception of the DMSO concentrations, and with the addition of Medium A concentrations which the isolates are suspended in. At the completion of suitable expression systems, this natural product library could be screened for possible inhibitors, as they have already been assayed for drug resistance and found to contain multi-drug resistant species of actinomycetes (16). Upon expression of the cloned resistance genes, the *B. subtilis* or *E. coli* cells would be able to grow in the presence of sub-MIC levels of antibiotic. However, the presence of a molecule that effectively inhibits the resistance mechanism would allow the efficacy of the antibiotic to be renewed and the cells would be killed. Therefore, a possible inhibitor would be identified as a compound that inhibits the growth of these cells, which express a resistance gene, at a sub-MIC level of the corresponding antibiotic. A possible positive control for the efficacy and efficiency of this screen could be the inhibition of the β-lactamase enzyme by clavulanic acid and tazobactam. As well, an additional positive control could be the use of the bacterial supernatant produced by *Streptomyces clavuligeris,* containing the natural source of clavulanic acid in proper growth conditions. In the event that none of the compounds successfully inhibit growth

under these conditions, an alternate approach could be to use bacterial strains that have increased sensitivity to these antibiotics, such as a pump deficient strain, or additionally, to try higher concentrations of the compounds in the library, or to try a different compound library entirely. However, in the event of a positive hit for an inhibitor, further experiments to characterize the compound would then be performed in hopes of finding a new inhibitor of an antibiotic resistance mechanism.

4.2 – Conclusion

Although both the pSWEET and pETcoco expression systems possess features which would be ideal for screening for inhibitors of antibiotic resistance, in a cell-based context, they are not faultless. Difficulty was encountered when attempting to integrate pSWEET fragments into *B. subtilis,* therefore the resistance gene was not present as a single-copy in the microorganism. Additionally, pETcoco was unable to generate consistent results in the optimization stage of the screen. Since sensitivity and reproducibility are crucial to the success of such a screen, the expression system employed must be extremely precise and efficient in order to yield significant results. With that said, it is clear that both the pSWEET and pETcoco expression systems, at this stage of progress, are unfit to serve as the expression systems of the proposed screens for inhibitors of antibiotic resistance.

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