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Effects Of Long-Term Exposure To Kanamycin and/or Ampicillin On Resistance Genes On An E. Coli Plasmid

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EFFECTS OF LONG-TERM EXPOSURE TO KANAMYCIN AND/OR AMPICILLIN ON RESISTANCE GENES ON AN *E. COLI* PLASMID

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PREFACE

This thesis is written in the style required by The American Society for Microbiology for the Journal of Cellular and Molecular Biology, to which a portion will be submitted for publication.

ABSTRACT

Multi-drug-resistant bacteria are a major cause of hospital-acquired infections and antibiotic resistance in these organisms is often plasmid mediated, which has become a growing concern. Plasmids conferring resistance to multiple antibiotics are increasingly becoming a common source of antibiotic resistance. The behavior of these plasmids under and in the absence of selective pressure is not yet fully understood. Therefore, to determine the behavior of a multiple-resistance plasmid under selective pressure pCR 2.1-TOPO, a commercial plasmid, was inserted into an *Escherichia coli* host and grown in a continuous culture under four conditions: broth with 1) kanamycin alone, 2) ampicillin alone, 3) with both kanamycin and ampicillin, and 4) without antibiotics. Samples were taken every two weeks, frozen, and later cultured on a replica plate series to identify mutants whose plasmids no longer conferred resistance to one or both antibiotics. The plasmids of these mutants were isolated, sequenced, and compared. The sequence data were analyzed to determine how the plasmid-mediated resistance genes changed over time. These results show the effects of selection pressure on the plasmid itself rather than on the organism by antibiotics and relates to the overall problem of antibiotic resistance in medicine and animal science by contributing to the understanding of the persistence of resistance markers in pathogen populations.

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INTRODUCTION

Multi-drug-resistant bacteria are a major cause of hospital-acquired infections and antibiotic resistance in these organisms is often plasmid mediated, which has become a growing concern[1]. Plasmids conferring resistance to multiple antibiotics are increasingly becoming a common source of antibiotic resistance[2]. The behavior of these plasmids under and in the absence of selective pressure is not yet fully understood[3]. To better understand this behavior this study examined the effects of long-term exposure to antibiotics on plasmid-mediated antibiotic resistance genes.

The plasmid pCR 2.1-TOPO (Figure 1) carries genes conferring resistance to kanamycin *via* aminoglycoside 3′-phosphotransferase and ampicillin *via* penicillin-binding protein 1A. The TOP10 (Invitrogen, Carlsbad, CA) strain of *Escherichia coli* was transformed with the pCR 2.1-TOPO plasmid and isolated using blue/white screening. From this isolated colony, four continuous cultures were established in Luria-Bertani (LB) broth media with kanamycin alone, ampicillin alone, both antibiotics, or without antibiotics at concentrations known to inhibit the growth of susceptible TOP10 *E. coli*. Samples of the continuous cultures were taken and frozen every two weeks and the minimum inhibitory concentration (MIC) later determined using Etest strips for each sample to kanamycin and ampicillin. To identify mutations that caused changes in MIC, each saved sample was cultured on a replica plate series and the MICs determined for the unique colonies isolated. The plasmids of these cultures were isolated and shipped to GeneWiz (South Plainfield, NJ) for nucleotide sequencing. The sequence data were analyzed using the Molecular Evolutionary Genomic Analysis (MEGA) tool. Lastly,

resistance to antibiotics to which the isolated samples were previously susceptible was regained by culturing the samples in sub-inhibitory concentrations of the antibiotics.

Introduction to Plasmid Biology

Conjugation is the most common and powerful method of horizontal gene transfer, in which plasmids are transferred between individual bacteria[4]. Plasmids, which are small, autonomous, extra-chromosomal pieces of DNA, can be considered the trading cards of bacterial cultures. Bacterial chromosomes contain the basic genes necessary for the proper growth and replication of the cell. These genes are the tried-and-true tools bacterial species have used to survive for millennia and which have undergone little change over time.

In contrast, plasmids are distinctly separate from the chromosome and tend to contain genes that confer traits such as resistance to antibiotics and other substances, metabolism of complex compounds or the production of such molecules, virulence factors, and other miscellaneous properties that are usually only transiently valuable to the cell[5]. Like trading cards, these plasmids can be gained as they become valuable in environments in which they are needed or lost when their value no longer merits the cost incurred by carrying them[6]. Due to the numerous functions plasmids may confer upon their hosts, especially the ability to confer resistance to antibiotics, and their current use in the study of molecular biology, the study of plasmids is an important avenue of research. For the purposes of this study, plasmid biology is presented as the history, classification, replication control, replication, inheritance, dissemination, and the clinical importance of plasmids. The methods of replication control, replication, inheritance, and

dissemination of plasmids vary and therefore general patterns for these processes will be discussed before discussing the method used by the pCR 2.1-TOPO plasmid used in this study.

History of Plasmid Research

The term plasmid was coined by Joshua Lederberg in 1952 in the journal *Physiological Reviews*, in which he stated,

> "These discussions have left a plethora of terms adrift: pangenes, plastogenes, chondriogenes, cytogenes and proviruses, which have lost their original utility owing to the accretion of vague or contradictory connotations. At the risk of adding to this list, I propose plasmid as a generic term for any extrachromosomal hereditary determinant. The plasmid may be genetically simple or complex[7]."

Originally, the study of plasmids was limited to microbial geneticists who studied them as mechanisms of gene exchange, but upon the discovery of their role in the spread of antibiotic resistance, a much wider audience became interested in plasmids.

Plasmids were discovered during studies conducted in the 1940s and 50s that were examining recombination in *E. coli.* These studies found that sexual differentiation was attributable to the presence or absence of a transmissible factor called F (for fertility). These cells could be classified as either F^+ or F^- based on the presence or absence of the F factor. Invariably, it was found that the transfer of genetic information was unidirectional: F^+ cells transferred genetic information to F^- cells, which then became

F^+ themselves[7]. This pattern of transmission of plasmids through conjugation from cell to cell was soon discovered to be responsible for another property of microbes that came as a surprise.

From the very beginning of the use of antibiotics in medicine, laboratories had been able to isolate resistant strains that had arisen from mutations in the chromosome, generally arising from point mutations[8]. These strains arose relatively infrequently. In striking contrast, soon after the development of single antibiotic resistance in clinical settings, the appearance and rapid spread of multiple antibiotic resistance plasmids led to the study of plasmids that today has made them indispensable tools in molecular biology.

Plasmid Classification

In an attempt to organize the large number of plasmids that were being discovered, with all their functional diversity, a classification system based on the replication system of the plasmid, a property common to all plasmids, was developed. This classification system, known as incompatibility grouping, is based on the inability of two plasmids to co-exist stably in the same cell line if they share the same replication system as defined by their origins of replication[9]. Many plasmids produce a repressor that inhibits replication of the plasmid at sufficient concentrations or an inducer that is responsible for the induction of replication. This concentration varies from plasmid to plasmid, but is responsible for the determination of plasmid copy number[10]. Plasmids that share the same repressor system will affect each other's rate of replication by each producing the repressor until the concentration of repressors is high enough to inhibit replication. The total number of plasmids will be approximately the same as if a single

plasmid were present despite the presence of two distinct plasmids[11]. Upon fission of the cell, the distribution of plasmids of each type to the daughter cells occurs at random. This leaves the plasmid composition of each cell to chance, eventually producing daughter cells containing a single plasmid. This is only a general pattern; however, as some plasmids of the same incompatibility group may not have the same copy number, and, in such cases, the plasmid at the higher copy number will out-replicate their competitors and in other plasmids, partitioning systems for the distribution of plasmids between daughter cells exist. In contrast, plasmid pairs that are not in the same incompatibility group do not have repressors that inhibit replication of the opposite plasmid and their relative contribution to the extra-chromosomal gene pool and copy number are consistent from generation to generation[11]. This system for the classification of plasmids is not without its weaknesses. Some plasmids contain more than one origin of replication that interferes with the ability of incompatibility testing. Other plasmids may have mutations that cause otherwise-incompatible and evolutionarily related plasmids to be considered compatible[11]. Other methods utilizing molecular probes are able to determine the presence of multiple replicons and small mutations within the replicon in order to more accurately classify plasmids into incompatibility groups[9].

Control of Plasmid Replication

The replication of plasmids independent of their host chromosome is one of their defining characteristics; however, their actions are not orchestrated entirely without regard to the proceedings of their hosts. In order to persist within a population, the replication rate of the plasmid cannot be sustained at a rate greater than that of the host

cell to prevent the risk of imposing a detrimental cost upon the host. Conversely, the replication rate cannot be sustained at less than the rate of host cell division which would eventually produce cells that lack the plasmid. The mechanisms by which replication is controlled are, therefore, an important aspect of plasmid biology. The models that have been proposed to explain the controlled replication of plasmids can be divided into two categories: passive and active[12].

Passive control strategies are those in which the plasmid copy number is limited by an external constraint. This may be a host-encoded protein that is essential for the initiation of replication[13]. In light of the incompatibility groups discussed previously, it is unlikely that a single host-encoded factor could be responsible for the regulation of the replication of plasmids. However, in natural environments with limited nutrient availability, passive control of plasmid replication due to the limitations of the cell's resources can influence the plasmid replication rate and copy number[12].

For active control systems, control of the rate of plasmid replication is determined by genes encoded within the plasmid itself. Two model systems for the control of replication rates have been proposed: the inhibitor dilution model and the autorepressor model, which differ in the encoded replication control gene being either a repressor or initiator of replication[14]. In the inhibitor dilution model, replication inhibitors are produced at a concentration proportional to plasmid copy number. The inhibitors function by binding to the origin of replication. Some inhibitors, instead of binding to the origin of replication, will bind to plasmid encoded initiators to inhibit the synthesis of proteins necessary for the initiation of replication. At a low plasmid copy number, inhibitor concentration is also low, which leaves the origin or initiator predominately unbound by

inhibitors, and replication of the plasmid is able to proceed. At higher copy numbers, inhibitor concentration is also higher and results in the inhibitor binding to the origin or initiator, thereby preventing further replication of the plasmid until the inhibitor concentration decreases when the cell either grows larger or divides[14].

In the autorepressor model, replication of the plasmid is meditated by an initiator protein that binds to the origin of replication and initiates replication. Within the operon coding for the initiator is also an autorepressor that is co-transcribed with the initiator. The autorepressor regulates the rate of transcription of both proteins by binding to the promoter-operator region of the autorepressor/initiator operon, and thereby maintains a constant concentration of both proteins. In some cases the autorepressor and initiator are a single bi-functional protein[15].

The plasmid pCR 2.1-TOPO contains the pUC origin of replication which is a derivative of the pMB1 origin of replication. The pMB1 origin does not require plasmid-encoded functions for its replication. It instead relies on replication enzymes produced by the host cell[16]. The pUC origin of replication within the pCR 2.1-TOPO plasmid also requires host enzymes for replication; however, the loss of the *rop* gene, responsible for regulation of the plasmid copy number, and a mutation in the *rep* gene, responsible initiation of replication, result in a copy number of 500-700 plasmids per cell[17]. This property is useful for the purposes of this study as the aim is to determine the effects of long-term exposure to antibiotics on the resistance genes themselves. Complications arising from mutations to other plasmid-encoded proteins are thereby avoided. However, this large copy number also makes the detection of mutations within a single plasmid extremely hard to isolate unless the plasmid with the mutation becomes the dominant

plasmid within the cell, as the phenotype of the bacteria is determined by the sum of the actions of the total plasmid population within that bacterial cell. With few plasmids carrying the mutation that would make the cell susceptible to an antibiotic, it is not until many generations after the mutation arises that it could be observed.

Plasmid Replication

The replication of plasmids is controlled, as discussed previously, at the level of initiation in a manner specialized for the maintenance of plasmids at an appropriate copy number; however, once replication is initiated, it proceeds in a manner very similar to that of chromosomal replication. Plasmid replication begins with the binding of a Rep protein, specific for the replicon present in the plasmid, to the interon sequence located near the origin of replication. Interons are short, repeated DNA sequences that, when bound by the Rep protein, separate the adjacent DNA sequence high in adenine and thymine[12]. This allows DNA helicase enzymes to enter the melted portion and further unwind the DNA template to reveal sites for the binding of the primer and the initiation of replication. The Rep protein and helicase enzymes are referred to as the pre-priming complex. Once the primer site is revealed, a primase enzyme synthesizes an RNA primer. The RNA primer is then extended with DNA by the enzyme DNA polymerase III in the leading and lagging strands[10].

There are a few special mechanisms for plasmid replication that are markedly different from the process described above. Rolling circle replication is a common form of replication in high-copy-number plasmids of *Staphylococcus, Lactobacillus, Mycoplasma, Streptococcus,* and *Streptomyces.* During rolling circle replication, the Rep

protein makes a single stranded break at the origin or replication while maintaining its attachment to the 5′-terminus of the break. The exposed 3′-terminus serves as a primer for the leading-strand DNA to be extended by DNA polymerase. RNA polymerase forms an RNA primer on the lagging strand at the palA locus, a point in the plasmid where a hairpin loop forms in the displaced strand. The lagging strand is then extended by DNA polymerase[18].

Another mechanism, called theta replication, in which there is no requirement for a plasmid-encoded Rep protein, is common in Col plasmids and other related multicopy plasmids. The initiation of theta replication begins with the transcription of a preprimer RNA approximately 555bp upstream of the origin of replication by RNA polymerase. The RNA transcript elongates as RNA polymerase travels towards the origin of replication and folds into a configuration which assists in the formation of a stable RNA-DNA hybrid at the origin of replication. Next, an endonuclease catalyzes the cleavage of the RNA strand of the RNA-DNA complex at the origin of replication, leaving behind what is essentially a primer, though not one produced by RNA primase. The 3′-terminus of the RNA transcript left behind after the cleavage of the RNA from the RNA-DNA complex is then extended by DNA polymerase. Downstream of the origin, unwinding reveals a primosome assembly site where lagging-strand synthesis is initiated[10].

The plasmid pCR 2.1-TOPO contains the pUC origin of replication. This origin of replication utilizes theta replication. As mentioned previously, the pCR 2.1-TOPO plasmid does not encode any of its own replication enzymes, and therefore uses the host cells RNA polymerase, endonucleases, DNA polymerase, and other enzymes required for

replication. The benefits and drawbacks of this with regard to this study were discussed previously.

Control of Plasmid Inheritance

For a plasmid to exist stably within a population, it must be inherited by both daughter cells reliably during cell division. This requires that the plasmids must replicate on average once per generation. Once the plasmid has replicated, the products of replication must be distributed between both daughter cells when the cell undergoes division. If this is not accomplished, a daughter cell receiving no plasmid DNA at cell division would give rise to a clone of plasmid-free descendants. There are different strategies used by high- and low-copy-number plasmids to deal with this issue.

Upon division of a bacterial cell, only extremely infrequently is there an instance in which one of the daughter cells does not receive a copy of the chromosome. This implies that there is a mechanism in place to ensure the equal distribution of

chromosomal DNA. For low-copy-number plasmids, similar mechanisms are required to ensure the inheritance of plasmids to daughter cells reliably. Two strategies to ensure the stable inheritance of low copy number plasmids have been proposed: equipartition and pair-site partitioning, though each assumes an association between the plasmid and cell membrane. In equipartition, half of the plasmids are inherited by each cell. In pair-site partitioning, a single pair of plasmids is partitioned into each daughter cell while the remaining plasmids are distributed randomly. For plasmids with a functioning copy number control system this is enough to ensure the continued presence of the plasmids within the cell line[19].

High-copy-number plasmids tend to segregate to daughter cells by random distribution. While this is unsuitable for low-copy-number plasmids, high-copy-number plasmids are able to be stably inherited by random distribution alone[19]. For a single plasmid, the probability of being distributed to each daughter cell is 0.5. For a high-copy-number plasmid, the probability of a single daughter cell receiving all of the plasmids present in 0.5^n, where *n* is the copy number of the plasmid. Since each cell divides into 2 daughter cells, the segregation frequency, or the probability that either one of the daughter cells will receive no plasmid, is $2(0.5)^{n\ 12}$.

The plasmid pCR 2.1-TOPO has a copy number of between 500 and 700 plasmids per cell and is distributed randomly to daughter cells[17]. At such a high copy number, the probability that the plasmid will not be inherited by both daughter cells is, for the purposes of this experiment, infinitesimal. That is not to say the presence of the pCR 2.1-TOPO plasmid is guaranteed once it enters a cell line. The metabolic load imposed on the host by the presence of the plasmid, such as the increase in generation time and use of cell components for replication, accelerates the rate of plasmid loss in the population. For the plasmid pCR 2.1-TOPO, the fitness cost imposed by the production of antibiotic-resistance products would, in cultures lacking antibiotics, form a situation in which the eventual loss of the plasmid would occur as cells lacking the plasmid out-compete their plasmid-bearing counterparts.

Methods of Plasmid Dissemination

Plasmids play an indispensable role in the evolution of bacterial species by providing a reservoir of genetic information transmissible between species. Plasmids can be transferred between cells in three ways: transduction, transformation, and conjugation.

Transduction occurs when a bacteriophage infecting a cell assembles its protein coat and bacterial chromosomal or plasmid DNA is inserted instead of phage DNA. The amount of DNA inserted is determined by the size and shape of the plasmid, with sizes similar to the phage genome being inserted most efficiently[12]. The phage protein behaves as if phage DNA were present and is able to bind to bacterial cells and insert the chromosomal or plasmid DNA. Once in the cell, plasmids need only regain their previous conformation to re-initiate autonomous replication. This method of horizontal gene transfer generally occurs in closely related species of bacteria, as the receptors for phage entry must be similar, and, therefore, it is likely that transduction is a less important means of gene transmission than conjugation, though transduction of plasmid DNA has been demonstrated in soil, fresh water, and waste water environments[20].

Transformation is the genetic alteration of a cell resulting from the uptake, integration, and expression of heterologous stretches of exogenous DNA from the surroundings through the cell membrane. Transformation is commonly used in molecular biology procedures to force cells to express foreign DNA; however, the procedures used in this process are not encountered in nature[20]. Natural transformation does occur and proceeds in several stages. The first stage is the acquisition of competence, or the ability to be transformed. This is accomplished in Gram-positive bacteria by accumulating a specific low-molecular-weight protein, a competence factor, from the local environment.

The next stage in transformation is the binding of DNA to the cell surface. Gram-negative species do not accumulate competence factors and, instead, undergo a period of unbalanced growth at the very end of the exponential phase of growth just before the stationary phase, during which the cells express genes for membrane-associated double stranded DNA binding proteins. Next, the DNA is transferred into the cell, and, finally, is integrated. The integration of plasmid DNA is easier than fragments of chromosomal DNA, as plasmids do not need to be integrated into the host chromosome to function[12].

The third mechanism of horizontal gene transfer, conjugation, is the transfer of plasmids between individuals *via* cell-to-cell contact. This gene exchange can occur between an enormous variety of bacterial species. A large number of conjugation systems have been identified in both Gram-negative and Gram-positive species. The plasmids that encode for their own conjugative factors are restricted by their size and tend to exist at low copy numbers to avoid an excessive metabolic cost to the host cell[12]. The process of conjugation begins with the synthesis of an extracellular pilus, essential for the maintenance of the intra-cellular environment that protects the plasmid DNA during transfer and for cell contact. These pili can be divided into those that are long (1μm) and flexible or short (0.1μm) and rigid. The type of pilus influences the rate of conjugation in different situations. After the pilus contacts the recipient cell, the pilus retracts, bringing the cells into close contact forming the DNA transport pore. Next, a single strand nick is introduced at the origin of transfer on the plasmid within the donor cell and the nicked strand is transferred to the recipient cell through the DNA transport pore. Once inside the recipient cell, the complementary strand is synthesized while the non-donor strand is also copied so the donor retains an intact plasmid[20].

The plasmid pCR 2.1-TOPO in this study has been transformed into the TOP10 strain of *E. coli,* which is a strain of *E. coli* lacking the fertility plasmid, and, therefore, cannot undergo conjugation. For the purposes of understanding how the antibiotic-resistance genes present are affected by long-term exposure to antibiotics, this property of the host *E. coli* is beneficial. The two factors that should determine the rates of bacteria carrying the pCR 2.1-TOPO plasmid are the random inheritance at cell division and the relative fitness of cells containing or lacking the plasmid.

Clinical Importance of Plasmids

Plasmids provide a large reservoir of highly mobile genes which, due to the restricted size of bacterial genomes, is utilized for the adaptation of bacterial species in changing environments[12]. These environments need not be natural, as demonstrated by one of the best documented examples for plasmid-driven evolution: the spread of antibiotic resistance.

Resistance to antibiotics was observed almost immediately after the introduction, mass production, and widespread use of most antibiotics. Typically, resistance was traced to spontaneous mutations on the bacterial chromosome. This was not seen as a cause for worry, as the probability of an individual bacterium obtaining mutations that would confer resistance to multiple antibiotics simultaneously was thought to be very low, and would only occur in environments where antibiotic exposure was common. To the surprise of many, transfer of antibiotic resistance between species was soon demonstrated with the transfer of multiple-antibiotic resistance from *Shigella flexneri* to *E. coli* and *Klebsiella pneumonia* in a mouse intestine in the absence of antibiotics[8]. The newly

discovered transfer of multiple antibiotic resistance between species stimulated interest in the subject and it was discovered that this resistance was independent of the chromosome. Soon, the transfer of resistance was compared to the activity of F plasmids and the plasmid-borne nature of antibiotic resistance was discovered.

That is not to say that all antibiotic resistance is plasmid mediated. Methicillin resistant *Staphylococcus aureus* (MRSA), for example, gains resistance to methicillin *via* a mutation in the gene encoding the penicillin binding protein. Further accumulation of resistance determinants from transposons led to the resistance of MRSA to many other antibiotics, leading to its current notoriety in clinical settings[21]. Despite this, antibiotic resistance plasmids are an important source of antibiotic resistance. In studying antibiotic resistance plasmids, it is important to understand how plasmids gain resistance genes, how these plasmids spread among bacterial species and interact with human populations, and how these genes persist within populations that are no longer exposed to antibiotics.

Unlike the point mutations that resulted in methicillin resistance in *Staphylococcus aureus,* the acquisition and loss of resistance genes on plasmids occurs as whole genes and gene blocks are reshuffled within plasmids and the host chromosome, generally due to the actions of transposons. Transposons come in two classes, known as Class I and Class II transposons. Class I transposons are also referred to as "copy-and-paste" transposons as they copy themselves and are inserted into a new position. Class II transposons are also known as "cut-and-paste" transposons. These transposons are able to excise themselves from the sequence of DNA and insert in a new place[12]. Regardless of which type of transposon is present, when a transposon is inserted on both sides of an antibiotic resistance gene, it forms a composite transposon that is able to be copied or

excised as a whole, mobilizing the resistance gene. Once this composite transposon is inserted into a plasmid, it becomes an antibiotic-resistance plasmid. If a resistance gene is already present on the plasmid, a multiple resistance plasmid is formed. These transposons can also move from plasmid to plasmid, making the development of multiple antibiotic resistance plasmids even more rapid, especially in the presence of high levels of antibiotics selecting for the presence of the resistance genes[22].

Antibiotic-resistance genes are transferred to populations of pathogens that interact with human populations in a variety of ways. The significant selective pressure exerted on antibiotic resistance genes in healthcare settings, the agricultural use of antibiotics, and ecological exposure to antibiotics all contribute to the transmission of antibiotic-resistance genes from the non-pathogenic environmental organisms and contribute to the selection pressure for antibiotic-resistance genes to persist in microbial populations.

Antibiotic-resistance genes are commonly found in hospital settings and, once introduced, these genes are known to spread rapidly[23]. There are several properties of the environment of hospitals that influence this rapid spread of resistance genes. Many of the bacteria that cause problems in hospitals are those that are present even in healthy individuals, however, when these bacteria acquire resistance genes and antibiotic treatments kill off other normal flora, they can cause life-threatening infections as they proliferate to fill the now-empty niches of their competitors[24]. Meanwhile, the high rate of antibiotic use for treatment of infections, as well as for prophylaxis during surgical procedures, generates a high level of selection for resistant phenotypes. Once the resistant phenotypes are present, hospital staff may act as a vector for the transmission of

pathogens between patients. With patients constantly entering and leaving the hospital environment and hospital staff potentially transmitting resistant bacteria between patients, resistance genes are able to enter into the community and, due to the chronic nature of many illnesses, other hospitals if the patient is re-admitted while still carrying resistant bacteria[23].

It is estimated that over 70% of the antibiotics used in the United States are not used to treat infections, but as a growth enhancer for agricultural purposes[25]. These practices have been banned elsewhere due to the propensity of this practice to generate antibiotic resistance. Use of antibiotics for growth enhancement is at sub-therapeutic levels, that is, levels that are insufficient to kill the susceptible microorganisms. This allows the susceptible populations to gain resistance in a step-wise fashion utilizing small phenotypic changes over time, whereas therapeutic doses are high enough that a sudden, large phenotypic change is required[26]. This becomes a problem when resistance gained by agricultural organisms is transferred to human populations[27]. The use of avoparcin in the European Union is a prime example of this process at work. Avoparcin, an analogue of vancomycin, was used extensively in feed on poultry and pig farms. In the mid-1990s a study of the rates of vancomycin-resistant *Enterococci* (VRE) in Denmark showed that pigs and poultry on farms where avoparcin was used as a growth promoter were three times as likely to carry VRE. Even after the use of avoparcin was banned in 1997, the rates of VRE remained above the levels observed in areas that were isolated from the avoparcin use. Countries that were in contact with countries that used avoparcin also saw a rise in VRE isolates without actually using avoparcin themselves[28].

Many antibiotics are isolated from soil bacteria that naturally produce them. The restrictions on nutrient availability and space for soil microbes has led to fierce competition between species and these bacteria have evolved a large armament of chemical weapons we call antibiotics. In addition to the production of antibiotics, mechanisms of resistance have evolved in soil bacterial to the antibiotics produced by themselves and other species. Although not all are useful for the treatment of infectious diseases, antibiotics isolated from soil bacteria are an important source of antibiotics[29]. Streptomycin, the first aminoglycoside antibiotic, was isolated from the soil bacterium *Streptomyces griseus* and current searches for novel antibiotics are being conducted in several labs using soil bacteria[30]. The resistance genes found in soil bacteria are also an important source of the spread of resistance genes into human pathogens. A survey of the antibiotic resistome, the collection of all the antibiotic resistance genes and their precursors, of 480 species of soil bacteria showed that there were no antibiotics that were effective against all species and, on average, each species was resistant to 8 of the 18 antibiotics tested[31]. These resistance genes in soil bacteria, through the mechanisms discussed previously, are capable of being transmitted to human pathogen populations. This becomes a problem when clinical antibiotics are introduced into the environment. Many antibiotics are cleared from the human body unchanged and can eventually make their way to environmental bacterial populations[25]. As with agricultural use at sub-therapeutic levels, this environmental exposure to low levels of antibiotic can help to generate antibiotic resistance that can then be transferred to human populations.

The clinical, agricultural, and environmental exposure of antibiotics all form a feedback system to generate and spread antibiotic resistance. However, even when the

use of antibiotics is stopped, resistance can be retained in pathogen populations. Understanding the factors that contribute to the retention and loss of antibiotic resistance over time will continue to be paramount to the prevention of the spread and development of antibiotic resistance. The roles that clinical, agricultural, and soil systems play in the persistence and spread of resistance genes show that antibiotic-resistance genes tend to persist in populations even when exposure to the antibiotics themselves has been lowered to sub-inhibitory levels or removed completely. The reasons for this persistence are not fully understood but can be attributed to the physical linkage of resistance genes and evolutionary processes compensating for the burden or carrying resistance genes.

Under the pressure of selection by the use of antibiotics, resistance genes can form non-random associations that aid in the retention of antibiotic-resistance genes in the absence of the antibiotic. These genes can cluster on plasmids and conjugative transposons. If several antibiotic-resistance genes are clustered on a plasmid or conjugative transposon, the presence of only one of the antibiotics exerts enough selective pressure that the entire resistance cassette is maintained[32]. How these clusters of resistance genes react over time in exposure to antibiotic conditions is not fully understood.

Keeping in mind that antibiotic resistance genes impose a fitness cost upon the host bacteria when expressed, it is easy to assume that, in the total absence of antibiotics, the population would eventually revert to sensitivity to antibiotics as susceptible individuals are no longer burdened by the imposed fitness cost of producing antibiotics. Unfortunately, this is not always the case. Compensatory mutations in genes other than the resistance genes can ameliorate the cost of antibiotic resistance while retaining the

resistance genes; therefore, when compensatory mutations are coupled with resistance genes, a high level of fitness can be achieved relative to susceptible competitors. However, when the compensatory mutations are detrimental to fitness when lacking resistance genes, the loss of resistance in these individuals causes a reduction of fitness greater than those individuals in which the compensatory mutation has not occurred and the resistance genes are still present[32]. This can establish a one-way street wherein the evolution of antibiotic resistance is not difficult, and, in the presence of antibiotics, is necessary, but the loss of resistance imposes a greater fitness cost than retention of the resistance genes and, therefore, resistance is maintained despite the loss of selection pressure[33].

The activity of antibiotic-resistance genes in the absence of selective pressure is not yet fully understood and, to this end, I have chosen to subject a multiple-resistance plasmid to several antibiotic conditions to determine how these genes change over time.

MATERIALS & METHODS

Obtaining the Ancestral *E. coli*

The original Top10 *E. coli* (Invitrogen, Carlsbad, CA) for this project was transformed by Dr. Yasuhiro Kobayashi and obtained on a Petri dish containing Luria-Bertani (LB) media, Xgal, and the transformed *E. coli* sample, known as a blue/white screen. From this blue/white screen, a single blue transformed colony was selected and transferred to a new LB plate and streaked for isolation. A colony selected from the isolation plate was then placed into 5mL of LB broth with 50µg/mL of kanamycin and ampicillin and grown overnight at 37˚C while on a gyrorotatry shaker set at approximately 100 rev/min. All other overnight incubations were conducted in this manner unless otherwise stated. A 750µl sample of this overnight culture was mixed with 250µl of 60% glycerol and stored at -80ºC to serve as an ancestral record.

Establishment of Continuous Cultures

From the overnight culture, four continuous culture environments were established. Fifty µL of the overnight culture from the ancestral *E. coli* were added to 8 culture tubes containing 5mL of fresh LB media. The cultures differed in the antibiotic content and were designated Con A, Con B, KanA, KanB, AmpA, AmpB, KA A, and KA B. The replicate cultures were used as a backup in case a culture failed to grow or was unusable. The positive control group, Con A and Con B, contained no antibiotics. The kanamycin group, Kan A and Kan B, were cultured in 50µg/mL kanamycin. The ampicillin group, Amp A and Amp B, were cultured in 50µg/mL of ampicillin. The

negative control group, KA A and KA B, were cultured in 50µg/mL of both kanamycin and ampicillin.

Continuous Culture

At approximately the same time each day, the cultured *E. coli* were transferred to fresh media to simulate a continuous exposure to the antibiotics. This was accomplished by transferring 50µl of the previous day's culture to 5mL of fresh media containing the appropriate antibiotics. The distribution of antibiotics in the cultures was maintained throughout the entire experiment and, therefore, was the same as that used in establishing the continuous cultures. After the continuous culturing procedure was completed, the previous day's cultures were discarded and the new cultures were incubated at 37˚C and agitated *via* gyrorotary shaker at approximately 100 rpm.

Freezing Samples for Later Use

At two-week intervals, samples of the continuous culture were frozen for later use in MIC testing, replica plating, and gene sequencing. Sample preservation was carried out by mixing 750µl of overnight culture media containing the *E. coli* to 250µl of 60% glycerol. Samples were then labeled with the culture designation and date of storage and stored at -80˚C until needed for later use.

Minimum Inhibitory Concentration Determination

Etest strips (BioMerieux, Durham, NC) were used to determine the MIC of antibiotics on the frozen samples. Frozen samples were thawed and 50µl were added to

5mL of LB media and incubated at 37°C overnight. No antibiotics were added during this culturing. Approximately 24 hours later the samples were removed from the incubator. Using a 0.5 McFarland standard as a template for the required inoculum turbidity, varying amounts of the *E. coli* sample were added to a sterile 0.85% saline solution in a spectrophotometer cuvette until the absorbance matched the absorbance of the 0.5 McFarland standard. After the correct absorbance was reached, the cuvette was labeled and placed in a sealed container until all samples were standardized. At this inoculum density, a confluent lawn of growth was obtained. The amount of culture required to accomplish the suggested inoculum turbidity varied with each culture, likely due to variations in culture density from overnight incubation.

The inoculation of Petri dishes to produce a bacterial lawn was accomplished by dipping a sterile swab in the inoculum suspension, removing excess fluid by pressing the swab against the inside wall of the cuvette, and carefully streaking the plate in 4 directions, each approximately 45 degrees apart. The plates were then allowed to dry. Drying occurred primarily while the remainder of the Petri plates to be used were inoculated or, in the case of the final plates, while the previous plates had Etest strips applied to them.

On the face of the Etest strip is a label indicating the antibiotic present and a logarithmic scale used to determine the MIC once growth is visible on the Petri dish. The back of the Etest strips contain varying amounts of antibiotic corresponding to the scale on the face of the strip. To apply the Etest strip, it was grasped at the top with flame-sterilized forceps, its bottom touched to the Petri dish, and released to fall into proper position. By placing the Etest strips away from the center of the plate, two Etest strips can

be placed in an anti-parallel orientation to ensure the formed ellipses do not overlap to determine the MIC of two antibiotics on the same plate. After inoculation and application of the Etest strips, the Petri plates were stacked in an inverted position in sets of five and incubated at 37ºC overnight.

Approximately 24 hours later, once bacterial growth was visible to the naked eye, the MICs were determined by observing where the inhibition ellipse intersects the Etest gradient strip. When growth occurred along the entire strip then no inhibition had occurred and the MIC was reported as equal to or greater then the highest value on the Etest strip.

Sample Dilution and Replica Plate Series

Replica plating is a technique in which one or more secondary Petri plates containing a selective marker, in this case the presence or absence of a combination of antibiotics, are inoculated with colonies from a master plate in such as way that they retain their spatial orientation.

Initially a master plate was created. The master plate was produced in a nearly identical manner to the Petri plates used in Etest MIC testing with the exception of further dilution once the 0.5 McFarland standard inoculum densities were achieved. Frozen samples were thawed and 50µl were added to 5mL of LB media and cultured overnight. No antibiotics were added during this culturing. Approximately 24 hours later, the samples were removed from the incubator. Using the 0.5 McFarland standard as a template for the required inoculum turbidity, varying amounts of the *E. coli* sample were added to a sterile 0.85% saline solution in a spectrophotometer cuvette until the

absorbance matched the absorbance of the 0.5 McFarland standard. This inoculum was then further diluted to 1/1000th the original density with sterile 0.85% saline. The cuvette was labeled and placed in a sealed container until all samples were prepared. At this inoculum density, the plates formed between 200-300 colonies per plate after 24 hours.

The replica plate series consisted of 5 plates. Plate 1 was the master plate. Plate 2 contained Xgal and kanamycin. Plate 3 contained Xgal and ampicillin. Plate 4 contained Xgal, kanamycin, and ampicillin. The final plate, plate 5, was a control plate that contained Xgal, but no antibiotics. Transfer of colonies was accomplished by pressing the master plate upon a sterile velveteen-covered disk. The velveteen cloth retained an imprint of the colonies of the master plate. Next, plates 2-5 were sequentially and carefully inoculated by the velveteen cloth by pressing them onto the cloth while maintaining the orientation of the plate to match the master plate. The inoculated replica plate series was then placed into the 37ºC incubator overnight.

Replica Plate Mutant Identification

After plates 2-5 were cultured overnight, photographs were taken of each plate on a colony counting apparatus. The apparatus consists of a light, a magnifying glass, and a grid pattern to aid in counting colonies. The light used is indirect so that it does not cause a glare on the Petri dish. The photographs taken of each colony were then cropped, centered, and enlarged to highlight the center of the Petri plates. In a manner similar to a blink comparator used to identify celestial objects as they move against the background of stars, the pictures of plates 2 and 3 were rapidly oscillated. This rapid oscillation allowed for the easy identification of mutant colonies that were capable of growth in the

presence of one but not both antibiotics. Plate 4 was used as a confirmation of the ability to grow only in the presence of a single antibiotic, while plate 5 was a positive control.

Replica Plate Mutant MIC Determination

Etest strips were used to determine the MIC of the identified replica plate mutants. Colonies isolated in the replica plate mutant identification step were sampled *via* a sterile inoculation loop and then cultured in 5mL LB media without antibiotics and placed in the 37ºC incubator. Approximately 24 hours later, the samples were removed from the incubator. From this point forward, the procedure for the determination of the MIC of the replica plate mutants is identical to that used to determine the MIC of the frozen samples.

Plasmid Isolation

The preparation of plasmid DNA by alkaline lysis with SDS[34] used in this instance is an alternative to the Wizard Plus SV Miniprep that provided a high plasmid yield. This plasmid isolation procedure is initiated by inoculating 3mL of TB broth, containing the appropriate antibiotics at 50µg/mL, with 50µl of saved culture. For cultures saved during the continuous culture, the antibiotics used were the same as those used for the continuous culture. For the isolated mutants from the replica plate series, the antibiotic used was the same as the antibiotic the plate on which the mutant colony was found. The inoculated TB broth was then cultured overnight at 37°C with shaking at 100 rpm.

From the overnight culture, 1.5mL were transferred to a sterile 1.5mL microcentrifuge tube and centrifuged at 12000 rpm for 35 seconds. The supernatant was then decanted so that only the cell pellet remained. The pellet was stored for 10 minutes at -70°C. The pellet was thawed for 5 minutes at room temperature. The pellet was then re-suspended in 150µl of Solution 1 with vigorous vortexing. Three-hundred µl of Solution 2 were added and mixed by inversion until the cell suspension had cleared, which took approximately 5 minutes. Two-hundred-fifty µl of Solution 3 were added and mixed by inversion until no trace of yellow liquid remained. The microcentrifuge tube was incubated at -20°C for 15 minutes. This chilled microcentrifuge tube was centrifuged for 12 minutes at 13000 rpm.

The supernatant from this centrifugation was transferred to a new 1.5µl microcentrifuge tube and 750µl of cold (20°C) isopropanol added. The solution was mixed by inversion followed by incubation at -20°C for 10 minutes. The plasmid DNA was isolated by centrifuging at 12000 rpm for 5 minutes. The supernatant was discarded, and the pellet washed with 300µl of 70% EtOH. After washing, the EtOH was discarded and any remaining EtOH was removed by vacuum centrifugation. The plasmid DNA was re-suspended in 150µl of sterile deionized water and stored at -20°C[34].

Plasmid Sequencing

The sequencing for this project was conducted by GeneWiz (South Plainfield, NJ) using ABI 3730xl DNA Analyzers for capillary electrophoresis and fluorescent dye terminator detection. Florescent dye-terminator sequencing utilizes labeling of the chain terminator dideoxynucletide triphosphates, ddNTPs, with fluorescent tags, which permit

sequencing in a single reaction, rather than the four separate reactions used in labeled-primer reaction methods. Each of the four ddNTPs is labeled with a florescent dye that emits light at a different wavelength. As the sample DNA is separated by size during capillary electrophoresis, the fluorescently labeled ddNTPs pass through a laser that causes the fluorescent tags to fluoresce. The wavelength of the resulting fluorescence is detected by a fluorescence detector and recorded. Initially during the separation of fragments of DNA and detection of fluorescence, the wavelength peaks tends to be very close together and determining the correct order of bases is not possible but can be done manually. For this experiment, the sequence obtained from this florescent dye-terminator sequencing is then used in primer walking to obtain the sequence of the whole pCR 2.1-TOPO plasmid. Primer walking is often used for sequencing DNA fragments between 1.3 and 7 kilobases as the fragments are too long to be sequenced in a single read using the chain-termination method. This method divides the sequence into several short sequences, each used to design a primer for the next portion of the sequence which were then used to form a consensus sequence that represents the total DNA fragment.

Bioinformatics Software Analysis of Sequence Data

The bulk of the previously discussed work was aimed towards the eventual identification of mutants susceptible to a single antibiotic, isolation of the plasmid responsible for this phenotype, and the acquisition of the nucleotide sequence of this plasmid. From these obtained sequences, analysis of the plasmid using bioinformatics software was possible. Using the Alignment Explorer tool within the Molecular Evolutionary Genomic Analysis (MEGA) program, the sequences of the manufacturers

pCR 2.1-TOPO plasmid, the ancestral pCR 2.1-TOPO, and the (Kan) Kan A 12-2-11 were aligned. This alignment was performed using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) method with a gap penalty of -20 and a gap extension penalty of -5. The obtained sequences were also mapped using the PlasmaDNA program.

Induction of Resistance in Previously Susceptible *E. coli*

The *E. coli* isolated during the replica plate procedure, from here forward referred to as KanR and AmpR, were determined to be susceptible to the antibiotics to which they were not exposed during the continuous culture procedure. These *E. coli* were used to inoculate LB broth cultures containing the antibiotic they were deemed susceptible to during the MIC determination of replica plate mutants. The KanR *E. coli* was used to inoculate 4 broth cultures containing ampicillin at 2µg/mL, 10µg/mL, 25µg/mL, and 50µg/mL. The AmpR *E. coli* was used to inoculate 4 broth cultures containing kanamycin at 2µg/mL, 10µg/mL, 25µg/mL, and 50µg/mL. These cultures were then incubated overnight. The culture with the highest concentration of antibiotics in which growth was observed was used to inoculate 4 new cultures with the same antibiotics at the same concentrations. This process was repeated until resistance growth was observed in all 4 concentrations of antibiotics for both cultures.

RESULTS & DISCUSSION

Minimum Inhibitory Concentration Determination

MICs were determined for the samples frozen throughout the experiment using Etest strips. The results for the MIC determination for kanamycin and ampicillin are represented in Tables 1 and 2. These MICs were determined during the week of 8 July 2012.

Between the 2nd and 4th weeks of the continuous culture in the positive control group, the activity of the plasmid to confer resistance to kanamycin and ampicillin drastically changed. By the 6th week, all resistance to ampicillin was lost in the positive control group and did not vary significantly throughout the rest of the experiment. Kanamycin resistance remained at a level below the concentration used during the continuous culture, ranging between 24 and 64µg/mL, but did not drop to the baseline for Top10 *E. coli* lacking the pCR 2.1-TOPO plasmid, whose MIC for both kanamycin and ampicillin was determined to be 2µg/mL. The reason for the elevated level of resistance above the baseline for Top10 *E. coli* has not yet been elucidated; however the determined MIC could have been affected by the amount of *E. coli* used to inoculate the plates used in the MIC determination procedure or the period of time for which the plates were incubated.

For all treatments other than the control treatment, the MIC of kanamycin and ampicillin remained at >256µg/mL throughout the duration of the experiment. This is likely due to the rarity of mutations that would confer lowered resistance to these antibiotics and the difficulty in detecting these mutants among their resistant cohorts.

The results of this procedure demonstrate that the pCR 2.1-TOPO multiple-resistance plasmid, when exposed to a single antibiotic, retains resistance to both antibiotics present in the vast majority of the *E. coli* carrying the plasmid. However, like other plasmids, when no antibiotics are present for a sufficient period, the plasmid is lost, as it no longer confers an advantage to the host.

Replica Plate Mutant Identification and MIC Determination

The plates produced in the replica series from the dilution of frozen samples were evaluated for the presence of colonies that were no longer resistant to both antibiotics. Fifteen bacterial colonies were isolated that, upon examination using the methods outlined above, appeared to grow in the presence of a single antibiotic. These 15 samples were added to LB media lacking antibiotics and grown overnight and then frozen for later MIC testing.

The 15 samples isolated during the identification of mutants that were able to grow in only the presence of a single antibiotic were cultured in order to determine the MICs to kanamycin and ampicillin using Etest strips. The results of the MIC determination of mutants identified using the replica plate series are listed in Table 3. Although 15 colonies were originally identified as mutants, only 2 of the colonies, when re-cultured and tested for MIC to kanamycin and ampicillin, showed susceptibility to a single antibiotic as indicated by the replica plating procedure. These samples are listed as KanR and AmpR in Table 3. This procedure demonstrated that, within a large population of resistant *E. coli* carrying the pCR 2.1-TOPO plasmid, there will be individuals who eventually lose resistance to an antibiotic to which they are no longer exposed.

Bioinformatics Software Analysis of Sequence Data

The sequences obtained *via* the primer walking process from GeneWiz were aligned with the manufacturer's stated sequence for the pCR 2.1-TOPO plasmid as well as to each other. GeneWiz was able to determine the sequence of the Ancestral 11/4/11 plasmid as well as the sample (Kan) Kan A 12/4/11, designated as KanR. The Alignment Explorer tool within the MEGA program was used to align the sequences. The sequences obtained from GeneWiz showed a 100% identity with each other; however, when compared to the sequence provided by the manufacturer there were gaps in the multiple cloning site. Upon further examination, it became clear that this gap was caused by the usage of the EcoR1 restriction enzyme being used with this plasmid. The portion of the alignment showing these gaps is located in Figure 2. This is unlikely to be the cause for the phenotypic change in the isolated *E. coli,* as the multiple cloning site is used for inserting genes of interest into the pCR 2.1-TOPO plasmid and the antibiotic resistance genes were both over 1300 bases upstream. This procedure demonstrated that, despite the presence of the plasmid and the appropriate resistance gene, colonies may be susceptible to an antibiotic which they have not been exposed.

Induction of Resistance in Previously Susceptible *E. coli*

The KanR and AmpR *E. coli* isolated on the replica plate were used to inoculate LB broth cultures containing the antibiotic to which they were previously deemed susceptible during the MIC determination of replica plate mutants. The KanR and AmpR *E. coli* were used to inoculate 4 broth cultures containing ampicillin or kanamycin at 2µg/mL, 10µg/mL, 25µg/mL, and 50µg/mL and the results of this procedure are listed in

the Day 1 row of Table 4. The culture with the highest concentration of antibiotics in which growth was observed, from the 2µg/mL culture of the KanR *E. coli* and the 25µg/mL culture of the AmpR *E. coli,* were used to inoculate 4 new cultures with the same antibiotics at the same concentrations. After overnight incubation, the Day 2 cultures were capable of growth in all four antibiotic concentrations. This procedure demonstrated that susceptible *E. coli* containing the appropriate resistance gene can quickly regain resistance upon exposure to the appropriate antibiotic.

Discrepancies between Hypothesized and Actual Results

Initially, I had hypothesized that, after 6 months of exposure to a single antibiotic, the vast majority of the *E. coli* present in the culture would have become susceptible to the antibiotic to which it had not been exposed through accumulated mutations in the resistance gene that was not under selective pressure to be maintained. Through the MIC determination of the saved cultures, it is obvious that this did not occur, as all samples in which any antibiotics were present retained resistance to both antibiotics. Further contradicting this hypothesis, the isolation of mutants that were resistant to a single antibiotic, while susceptible to the antibiotic to which they were not exposed showed that these mutants were rare within the populations and when the sequences of the plasmids of the isolated *E. coli* were compared to the sequence of the plasmid used to start the continuous culture, the resistance genes had not accumulated any mutations. The reasons for this behavior are not clear; however, I believe it is possibly due to linkage of the resistance genes, compensatory mutations, or the very high copy number of the pCR 2.1-TOPO plasmid.

CONCLUSIONS

This study aimed to examine the effects of long-term exposure to antibiotics on plasmid-mediated antibiotic-resistance genes. This was achieved by continuously culturing *E. coli* containing a multiple-resistance plasmid in different antibiotic combinations. The MIC of these cultures was determined at regular intervals and upon finding no change in the MIC of cultures regardless of the antibiotic to which they were exposed, as long as they were exposed to an antibiotic, a replica plating procedure was used to isolate any mutants that were resistant to a single antibiotic. The *E. coli* isolated during this procedure, KanR and AmpR, as well as the plasmid from the *E. coli* used to start the continuous culture, had their plasmids isolated. The isolated plasmids were shipped to GeneWiz so that the nucleic acid sequences could be determined using primer walking. The obtained sequences were compared showing that the kanamycin and ampicillin resistance genes were still present in both of the isolated samples and were identical to the genes present before the continuous culture procedure. These samples were then cultured in the presence of the antibiotic to which they were deemed susceptible in order to induce resistance to the antibiotic.

In summary, this experiment has shown that multiple-resistance plasmids tend to retain resistance to any antibiotics to which the plasmid confers resistance as long as one of the antibiotics in question is present. In a relatively small number of individuals, the expression of the resistance gene is reduced and may ultimately be stopped when the corresponding antibiotic is not present for long periods of time; however, upon re-

exposure to the antibiotic, expression of the gene and resistance to the antibiotic are quickly regained.

To improve upon this project, several changes could be made. First, using a plasmid with a lower copy number would aid in identifying colonies in which with mutations have arisen or expression of the resistance gene has been reduced. Second, using sub-inhibitory concentrations of antibiotic as one of the treatments during the continuous culture would be useful in determining if the plasmid would be retained and in its original form when antibiotics are present in sub-inhibitory concentrations. Third, using a plasmid that confers resistance to more than 2 antibiotics would help in determining the exact relationship between the genes present and how the maintenance of one gene affects the others. Fourth, using minimally nutritious media in order to increase the relative cost of carrying the plasmid would aid in making the plasmid a burden on the host so that the plasmid and its resistance genes would be more likely to mutate and become non-functional as a mechanism of ameliorating the associated fitness cost. Finally, using a plasmid whose resistance genes are farther apart would help in determining the role the spatial arrangement of the resistance genes on the retention of resistance genes when the antibiotic is no longer present.

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FIGURES

Figure 1. pCR 2.1-TOPO plasmid map generated with PlasmaDNA of manufacturer's provided sequence.

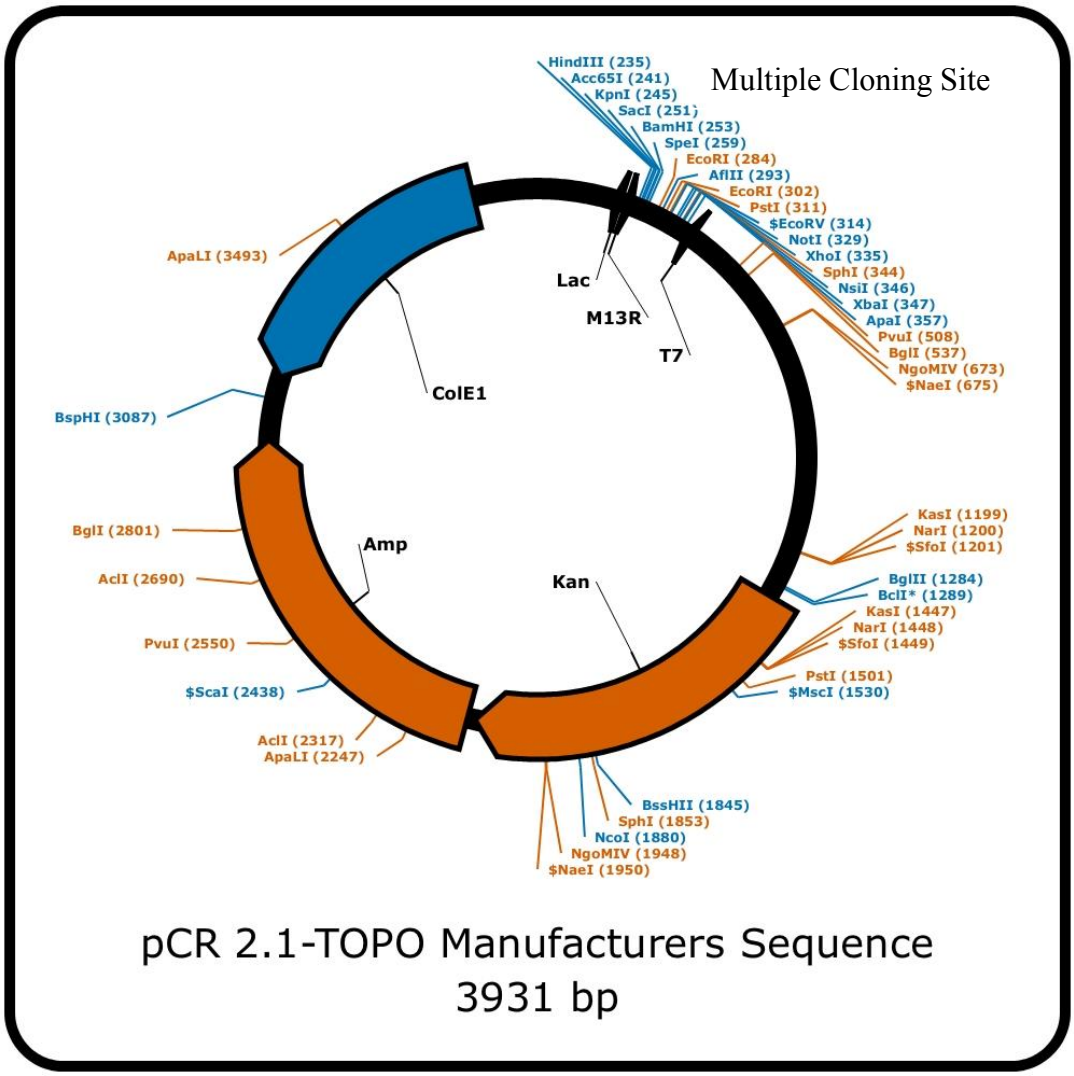
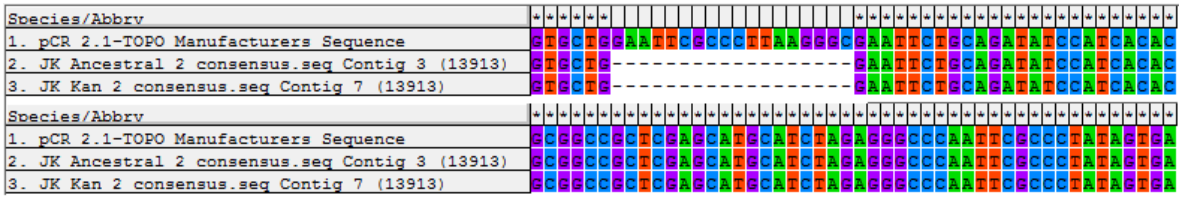


Figure 2. MEGA alignment results of the manufacturer's stated sequence of the pCR 2.1-TOPO plasmid with the reported sequence from GeneWiz for the KanR and Ancestral plasmids showing the gaps located in the multiple cloning site.



TABLES

Table 1. Minimum inhibitory concentration of kanamycin for each treatment over the course of six months in µg/mL. Asterisks (*) signify that colonies were present in the formed ellipse. For colonies in which no ellipse was formed, resistance was reported as >256µg/mL.

	MIC tested against Kanamycin							
	No antibiotics		Kanamycin		Ampicillin		Kan and Amp	
Date Saved	**Control A**	**Control B**	**Kan A**	**Kan B**	**Amp A**	**Amp B**	**KA A**	**KA B**
11/4/2011	>256	>256	>256	>256	>256	>256	>256	>256
11/18/2011	>256*	>256*	>256	>256	>256	>256	>256	>256
12/2/2011	24*	48*	>256	>256	>256	>256	>256	>256
12/16/2011	32	64*	>256	>256	>256	>256	>256	>256
12/30/2011	32	48	>256	>256	>256	>256	>256	>256
1/13/2012	24	48	>256	>256	>256	>256	>256	>256
1/31/2012	64*	64	>256	>256	>256	>256	>256	>256
2/14/2012	32	48*	>256	>256	>256	>256	>256	>256
2/28/2012	48	48	>256	>256	>256	>256	>256	>256
3/13/2012	64	96	>256	>256	>256	>256	>256	>256
3/27/2012	48	32	>256	>256	>256	>256	>256	>256
4/10/2012	32	32	>256	>256	>256	>256	>256	>256
4/23/2012	64	64	>256	>256	>256	>256	>256	>256

Table 2. Minimum inhibitory concentration of ampicillin for each treatment over the course of six months in μg/mL. Asterisks (*) signify that colonies were present in the formed ellipse. For colonies in which no ellipse was formed, resistance was reported as >256μg/mL.

	MIC tested against Ampicillin							
	No antibiotics		Kanamycin		Ampicillin		Kan and Amp	
Date Saved	**Control A**	**Control B**	**Kan A**	**Kan B**	**Amp A**	**Amp B**	**KA A**	**KA B**
11/4/2011	>256	>256	>256	>256	>256	>256	>256	>256
11/18/2011	>256*	>256*	>256	>256	>256	>256	>256	>256
12/2/2011	2*	4*	>256	>256	>256	>256	>256	>256
12/16/2011	3*	4*	>256	>256	>256	>256	>256	>256
12/30/2011	2	3	>256	>256	>256	>256	>256	>256
1/13/2012	0.5	1	>256	>256	>256	>256	>256	>256
1/31/2012	1.5*	1.0*	>256	>256	>256	>256	>256	>256
2/14/2012	2	2*	>256	>256	>256	>256	>256	>256
2/28/2012	4	2	>256	>256	>256	>256	>256	>256
3/13/2012	4	3	>256	>256	>256	>256	>256	>256
3/27/2012	4	2	>256	>256	>256	>256	>256	>256
4/10/2012	2	3	>256	>256	>256	>256	>256	>256
4/23/2012	3	3	>256	>256	>256	>256	>256	>256

Table 3. Minimum inhibitory concentrations of kanamycin and ampicillin for samples isolated from the replica plate series. For colonies in which no ellipse was formed, resistance was reported as >256µg/mL.

	Replica Plate Mutant MIC Results	
E. coli	**Kanamycin Resistance (µg/ml)**	**Ampicillin Resistance (µg/ml)**
KanR	>256	2
AmpR	2	>256

Table 4. Induction of antibiotic resistance in previously susceptible *E. coli* after overnight exposure to antibiotics showing growth as + or – for a given culture. KanR *E. coli* is the (Kan) KanA 12/2/11 sample obtained in the replica plate procedure. AmpR *E. coli* is the (Amp) AmpB 3/31/12 sample obtained in the replica plate procedure.

Induction of Antibiotic Resistance					
		Antibiotic Concentration in Broth			
Day	***E. Coli***	**10µg/mL**	**25µg/mL**	**50µg/mL**	**100µg/mL**
1	KanR *E. coli*	+	-	-	-
	AmpR *E. coli*	+	+	-	-
2	KanR *E. coli*	+	+	+	+
	AmpR *E. coli*	+	+	+	+

APPENDIX

Appendices. Sequences of pCR 2.1-TOPO plasmid provided by the manufacturer, from the colony used to start the continuous culture, and those isolated in replica plate series.

Appendix 1. pCR 2.1-TOPO Sequence from Manufacturer:

AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATG

CAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGC

AATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGC

TTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGA

AACAGCTATGACCATGATTACGCCAAGCTTGGTACCGAGCTCGGATCCACTA

GTAACGGCCGCCAGTGTGCTGGAATTCGCCCTTAAGGGCGAATTCTGCAGAT

ATCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCAATTCGCC

CTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACT

GGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTC

GCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGT

TGCGCAGCCTGAATGGCGAATGGACGCGCCCTGTAGCGGCGCATTAAGCGCG

GCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAG

CGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTC

CCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTA

CGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGC

CATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTT

AATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTA

TTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATG

AGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATTCAGGGCGCAAG

GGCTGCTAAAGGAAGCGGAACACGTAGAAAGCCAGTCCGCAGAAACGGTGC

TGACCCCGGATGAATGTCAGCTACTGGGCTATCTGGACAAGGGAAAACGCAA

GCGCAAAGAGAAAGCAGGTAGCTTGCAGTGGGCTTACATGGCGATAGCTAG

ACTGGGCGGTTTTATGGACAGCAAGCGAACCGGAATTGCCAGCTGGGGCGCC

CTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTTTCTTGCCG

CCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATGA

GGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCG

CTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTG

CTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTG

TCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCG

GCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTG

TCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGG

ATCTCCTGTCATCCCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGAT

GCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACC

AAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGT

CGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACT

GTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACC

CATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGG

ATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCG

TTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCT

TCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTAT

CGCCTTCTTGACGAGTTCTTCTGAATTGAAAAAGGAAGAGTATGAGTATTCA

ACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTT

GCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTG

CACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAG

TTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTAT

GTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCG

CATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAG

CATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCA

TGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAA

GGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATC

GTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCA

CGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACT

ACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAA

GTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGA

TAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGG

CCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGG

CAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGAT

TAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATT

TAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAAT

CTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCC

CGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCT

GCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGA

TCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAG

ATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAA

CTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTG

CTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTT

ACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCC

CAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTA

TGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTA

AGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAAC

GCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCG

ATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAAC

GCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTT

CCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGC

TGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGA

GGAAGCGGAAG

Appendix 2. pCR 2.1-TOPO Ancestral Sequence:

AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATG

CAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGC

AATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGC

TTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGA

AACAGCTATGACCATGATTACGCCAAGCTTGGTACCGAGCTCGGATCCACTA

GTAACGGCCGCCAGTGTGCTGGAATTCTGCAGATATCCATCACACTGGCGGC

CGCTCGAGCATGCATCTAGAGGGCCCAATTCGCCCTATAGTGAGTCGTATTAC

AATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTAC

CCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCG

AAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGA

ATGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGC

GCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTC

TTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGG

GGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAA

ACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTT

TTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAA

ACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGAT

TTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTA

ACGCGAATTTTAACAAAATTCAGGGCGCAAGGGCTGCTAAAGGAAGCGGAA

CACGTAGAAAGCCAGTCCGCAGAAACGGTGCTGACCCCGGATGAATGTCAGC

TACTGGGCTATCTGGACAAGGGAAAACGCAAGCGCAAAGAGAAAGCAGGTA

GCTTGCAGTGGGCTTACATGGCGATAGCTAGACTGGGCGGTTTTATGGACAG

CAAGCGAACCGGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCC

CTGCAAAGTAAACTGGATGGCTTTCTTGCCGCCAAGGATCTGATGGCGCAGG

GGATCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTCGCATGATTGAAC

AAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGG

CTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGG

CTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGC

CCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACG

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GGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCCCACCTTGCT

CCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGC

TTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCG

AGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAA

GAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCA

TGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAA

TATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGG

GTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGA

AGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCG

CTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA

ATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCC

TTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAA

GTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGG

ATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCA

ATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGA

CGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTG

GTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAA

GAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTT

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ATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAG

CCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAAC

GTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAAT

TAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGC

CCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGT

CTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGT

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GTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGG

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GTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTT

GAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCG

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CCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGC

TCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTA

CCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTG

AACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGA

ACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGG

AGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGC

ACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGT

TTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGG

AGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTG

CTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAA

CCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACC

GAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAG

Appendix 3. pCR 2.1-TOPO Sequence from (Kan) Kan A 12-2-11:

AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATG

CAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGC

AATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGC

TTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGA

AACAGCTATGACCATGATTACGCCAAGCTTGGTACCGAGCTCGGATCCACTA

GTAACGGCCGCCAGTGTGCTGGAATTCTGCAGATATCCATCACACTGGCGGC

CGCTCGAGCATGCATCTAGAGGGCCCAATTCGCCCTATAGTGAGTCGTATTAC

AATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTAC

CCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCG

AAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGA

ATGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGC

GCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTC

TTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGG

GGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAA

ACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTT

TTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAA

ACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGAT

TTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTA

ACGCGAATTTTAACAAAATTCAGGGCGCAAGGGCTGCTAAAGGAAGCGGAA

CACGTAGAAAGCCAGTCCGCAGAAACGGTGCTGACCCCGGATGAATGTCAGC

TACTGGGCTATCTGGACAAGGGAAAACGCAAGCGCAAAGAGAAAGCAGGTA

GCTTGCAGTGGGCTTACATGGCGATAGCTAGACTGGGCGGTTTTATGGACAG

CAAGCGAACCGGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCC

CTGCAAAGTAAACTGGATGGCTTTCTTGCCGCCAAGGATCTGATGGCGCAGG

GGATCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTCGCATGATTGAAC

AAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGG

CTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGG

CTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGC

CCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACG

GGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACT

GGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCCCACCTTGCT

CCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGC

TTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCG

AGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAA

GAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCA

TGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAA

TATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGG

GTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGA

AGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCG

CTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA

ATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCC

TTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAA

GTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGG

ATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCA

ATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGA

CGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTG

GTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAA

GAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTT

ACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAAC

ATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAG

CCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAAC

GTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAAT

TAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGC

CCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGT

CTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGT

AGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACA

GATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAA

GTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGG

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GTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTT

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ACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGG

AGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGC

ACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGT

TTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGG

AGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTG

CTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAA

CCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACC

GAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAG