

Development of a Recombineering System in *Enterobacter* sp. YSU

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ABSTRACT

Recombineering, also known as recombination-mediated genetic engineering, is a molecular genetics technique that utilizes homologous recombination to modify the genome of prokaryotes and eukaryotes *in vivo*. One recombination system is the lambda Red recombination system that is controlled by the lambda bacteriophage, which contains the *red* genes that encode the Exo, Beta, and Gam proteins. The Exo, Beta, and Gam proteins are involved in the process of double strand break repair and are responsible for homologous recombination. This method of recombination has replaced the more conventional and time-consuming genome modification technique using restriction endonuclease enzymes and DNA ligase. I hypothesize that the lambda Red recombination system will be successful in *Enterobacter* sp. YSU because it was developed in *Escherichia coli* and has been proven to be successful in *Enterobacter cloacae*, *Enterobacter aerogenes* and *Saccharomyces cerevisiae*. pKD46 plasmid was PCR amplified to remove the ampicillin resistance gene because *Enterobacter* sp. YSU is already resistant to ampicillin which is the selectable marker for pKD46.

A chloramphenicol and kanamycin resistance gene was PCR amplified and mixed with the pKD46 PCR product without the *ampR* gene, treated with T4 Polynucleotide Kinase, and ligated to construct two new plasmids, pKD46-cm and pKD46-kan. pKD46-cm and pKD46-kan were used for recombination with pBR322 plasmid to replace the *ampR* gene with chloramphenicol or kanamycin resistance. *AmpR* was successfully replaced with kanamycin resistance using pKD46-cm and with chloramphenicol resistance using pKD46-kan in *E. coli*. Recombination in *Enterobacter* sp. YSU via transformation was attempted using pKD46-kan to replace *cmR* with kanamycin

resistance because it was successful in *E. coli*. However, it was not successful because *cmR* from pACYCY184 does not appear to be expressed well in YSU. This recombination system can be useful in helping understand gene function by creating gene replacements, deletions, insertions, gene/protein tagging, and gene cloning.

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CHAPTER I: INTRODUCTION

1.1 Importance of Recombinant DNA Technology

Recombinant DNA technology has paved the way for innovative possibilities in genetics as well as provided opportunities in medicine, agriculture, and industry. This more modern system can improve medication efficacy, increase crop yields, lower medication costs, prevent genetic diseases and promote disease resistances in livestock and crops. (Berg & Singer, 1995).

Recombinant DNA technology can assist in helping to understand gene function through gene knockouts, interruptions and deletions. It also offers a means to understanding structure as well as provides many practical benefits for scientific applications (Tilghman et al., 1977). It dominates many areas of biology as well as diverse fields such as chemistry, evolution, psychology, plant science medicine, and forensics. The most insightful effect has been in the increased knowledge of essential life processes. Genes are no longer thought of as a theoretical belief. Now genes can be synthesized in test tubes, manipulated, and reintroduced into cells of diverse living organisms. This allows us to show relationships between genes and their unique physiological functions (Berg & Singer, 1995).

One application for recombinant DNA technology is in the manufacturing of medication. The possibility for an insulin shortage for the treatment of diabetes has become a major focus lately. A study by Keefer et al. (Keefer, Piron, & De Meyts, 1981) successfully synthesized the A and B chains of human insulin using synthetic cloned genes in *E. coli*. This recombinant insulin showed remarkable similarity to native

pancreatic human and porcine insulin in relation to binding affinity, association/dissociation kinetics, down regulation of receptors, and stimulation of lipogenesis.

The *lacZ* gene, which encodes the protein, β -galactosidase, that cleaves disaccharide lactose into glucose and galactose, has been used as a reporter gene because of its ease of function (Seier et al., 2011). The *lac* operon is required for lactose metabolism and transport in *E. coli*. It contains 3 structural genes, *lacZ*, *lacY*, and *lacA* which encode different proteins (Abdoli, Amirthalingam, Lillquist, & Nutt, 2007; Seier et al., 2011). *LacZ* encodes beta-galactosidase, *lacY* encodes lactose permease and *lacA* encodes galactoside O-acetyltransferase. O-nitrophenyl- β -d-galactopyranoside (ONPG) is used as an indicator of β -galactosidase activity by turning yellow when hydrolyzed by LacZ. Bacterial colonies containing β -galactosidase turn blue in the presence of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, also referred to as X-gal (Abdoli et al., 2007). Reporter genes have been essential in the analyses of gene interaction (Mansour, Thomas, Deng, & Capecchi, 1990).

1.2 Previous Recombinant DNA Technology Methods

Genetic engineering revolutionized molecular biology research for over 30 years by providing fundamental experimental methodology. Previous recombinant DNA technology used restriction endonucleases and DNA ligase which requires complicated targeting and selection constructs has been the primary method adopted to modify bacterial genomic DNA (Copeland, Jenkins, & Court, 2001; Yu et al., 2000). Restriction endonucleases are enzymes that cut DNA at specific locations called restriction sites. DNA ligase is an enzyme that catalyzes the formation of phosphodiester bonds, which

facilitates the joining of the DNA strands together. This system has been very useful in generating recombinant molecules. However, the location of these restriction sites is essential to the success of the recombination and most often the desired restriction site is not always available.

Availability of unique, specific restriction sites has complicated the propagation of these constructs. Requirement of large DNA constructs necessary for previous recombination techniques has proven inefficient due to the inability for cloning vectors to uptake these larger, less tolerable inserts (Copeland et al., 2001). Techniques such as recombineering facilitate the use of much shorter and more stable DNA homologies than previous conventional methods (Sawitzke et al., 2007; L. Thomason et al., 2007). To solve this problem, the target gene is amplified using the polymerase chain reactions (PCR) and primers containing 5' end linkers with the desired restriction endonuclease sites. Even with this PCR strategy, the use of restriction endonuclease and DNA ligase for cloning is only useful for constructs up to 10 kb in size. Recombination using restriction endonucleases and DNA ligase becomes fundamentally more difficult with larger DNA molecules (Berg & Singer, 1995; Narayanan & Chen, 2011).

1.3 Benefits of Recombineering

Conventional methods focus on recombination being performed *in vitro* which is much more time consuming than the more modern *in vivo* recombineering method (Court, Sawitzke, & Thomason, 2002) and is more accurate, efficient, and straightforward (Costantino & Court, 2003; Court et al., 2002; Sawitzke et al., 2007; Sharan, Thomason, Kuznetsov, & Court, 2009; Yu et al., 2000). Recombineering can promote retrieval of target sites for linear dsDNA cassettes, resident plasmids,

chromosomes, and genes from genomic DNA which make it more efficacious (Narayanan & Chen, 2011). Constructs too complicated for previous conventional methods can be created in a matter of days using recombineering which uses linear PCR products or single stranded oligonucleotides to promote homologous recombination (Sawitzke et al., 2007; Sharan et al., 2009; L. Thomason et al., 2007).

Recombineering is also capable of producing single base changes, deletions, and insertions in Bacterial Artificial Chromosomes (L. C. Thomason, Oppenheim, & Court, 2009). Bacterial Artificial Chromosomes (BACs) are F-plasmid vectors that allow cloning of DNA up to 300 kb (Karstentischer, von Einem, Kaufer, & Osterrieder, 2006; Sharan et al., 2009). BACs large size has restricted precise modifications as well as the introduction of reporter genes, mutations, and selectable markers for functional applications. Recombineering is not limited by size which permits more responsive modifications on BACs (Narayanan & Chen, 2011). Therefore, reporter genes can be inserted easier at the 5' and 3' ends of genes cloned in to BACs (Sharan et al., 2009).

The application of recombineering, which was originally restricted to smaller plasmids, has permitted genetic manipulation in larger constructs such as BACs (Copeland et al., 2001; Karstentischer et al., 2006; Sharan et al., 2009). BAC mutagenesis and cloning can be accomplished using recombineering due to the construction of their targeting substrate which may either be a resident plasmid, chromosomal region, or an alternate source of DNA and the incoming targeting substrate can be a linear dsDNA or single stranded oligonucleotide (Narayanan & Chen, 2011).

Recombineering has simplified the propagation of transgenic and knockout constructs which allows larger DNA segments to be engineered such as BACs

which replicate at low copy numbers in *E. coli* (Copeland et al., 2001; Sharan et al., 2009). This system has also increased efficiency of genetic engineering by allowing more complex modifications to be manipulated easier. Larger constructs such as BACs can now be directly altered (Copeland et al., 2001; Sawitzke et al., 2007). BACs are ideal for propagating transgenic organisms because their insert size allows expression of the controlled gene under the control of its own regulatory elements such as promoter, terminator, and enhancers (Sharan et al., 2009).

1.4 Red/ET System Precursors

Homologous recombination is highly dependent on RecA in *E. coli*. RecA allows ssDNA to bind and form protein filaments that locate specific sequence homologies on alternative DNA molecules. Once the sequence is located, RecA filament exchanges its strand with the complementary homologous strand. RecBCD, which requires RecA function, initiates recombination at dsDNA by generating 3' overhangs. RecA-dependent recombination and Rec-A independent phage mediated recombination differ due to their pairing and the way strand exchange occurs. RecBCD plays a major role in the recombination system in *E. coli* and is also dependent on the use of the RecA protein (Murphy, 1998; L. Thomason et al., 2007). RecBCD generates 3' ssDNA overhangs and helps RecA bind to this ssDNA. The binding of RecA to the overhang promotes strand exchange via homologous recombination. RecA independent functions are much more straightforward than RecA dependent functions (Court et al., 2002; Hillyar, 2012; Yu et al., 2000). In *E. coli*, recombination is primarily dependent on the use of a DNA repair by RecA. RecA forms filaments by binding to ssDNA fragments that have the ability to locate a homologous sequence on a DNA strand. Once a homologous sequence is located

and isolated, RecA filaments exchange strands with its complementary fragment (Court et al., 2002; L. Thomason et al., 2007).

In *Escherichia coli* (*E. coli*), linear dsDNA is unstable due to a RecBCD exonuclease, which cuts at 5' GCTTGGTGG 3' nucleotide sequences. *E. coli* strains that lack the RecBCD exonuclease due to a *recBC* mutation can be transformed by linear dsDNA. These mutant strains must also contain *sbcB* and *sbcC* mutants, which restore activity to *recBC* mutants to promote the allowance of recombination. These *recBC* mutant strains have provided very efficient *in vivo* cloning strategies in *E. coli* (Copeland et al., 2001; Yu et al., 2000).

1.5 Red/ET Systems

The lambda Red system that uses the Exo, Beta, and Gam recombination proteins was developed from the lysogenic lambda bacteriophage (Datsenko & Wanner, 2000; Lee et al., 2001; L. Thomason et al., 2007). This phage infects *E. coli* and inserts its DNA into a bacterial chromosome. To insert its DNA into the host, bacteriophage lambda contains a homologous recombination system entitled the lambda Red system which consists of the Exo, Beta, and Gam proteins (Sawitzke et al., 2007; L. Thomason et al., 2007; Yu et al., 2000).

An updated mechanism of action, states that a ssDNA intermediate is responsible for replication-fork annealing (Mosberg, Lajoie, & Church, 2010). Degradation on one of the strands of the dsDNA by Exo is the first step in recombination. Exo facilitates the binding of Beta to the single stranded intermediate. Beta binds to the ssDNA and protects it from degradation and catalyzes its placement while annealing it to the lagging strand of the replication fork. The oligonucleotide homology regions bind to the complementary

regions and in the next round of replication the genetic mutation is incorporated into the newly synthesized DNA (Boyle, Reynolds, Evans, Lynch, & Gill, 2013).

A unique function of Beta is that it can bind to single stranded fragments greater than 35 bp and protects DNA from nuclease attack. Therefore, it is the only function necessary for ssDNA oligonucleotide recombineering. However, all three functions remain necessary to carry out recombineering utilizing double stranded DNA (Chan et al., 2007; Costantino & Court, 2003; Ellis, Yu, DiTizio, & Court, 2001; Sharan et al., 2009). Linear DNA is usually degraded by the RecBCD nuclease (Sawitzke et al., 2007; L. Thomason et al., 2007). The Gam protein inhibits RecBCD nuclease activity by allowing the survival of linear DNA that can be used as a substrate for recombination (Chan et al., 2007; Oppenheim, Rattray, Bubunencko, Thomason, & Court, 2004; Sawitzke et al., 2007; Swingle et al., 2010; L. C. Thomason et al., 2009). Generating recombinants using the lambda Red system is productive even in the absence of RecA (Copeland et al., 2001; Court et al., 2002).

In the original construct, the CI-repressor, a temperature sensitive repressor, controls the expression of the prophage as well as the lambda Red functions (Copeland et al., 2001; Liu, Jenkins, & Copeland, 2003; L. Thomason et al., 2007; Yu et al., 2000). To obtain relatively efficient recombination of linear DNA, *E. coli* containing the lambda prophage that contains the Exo, Beta, and Gam red functions under the control of a CI-repressor was constituted. In this system, the *red* genes can be easily switched on at 42°C and switched off at 32°C (Yu et al., 2000). At 32 °C, the Red genes remain unexpressed due to the activation of the CI-repressor (Copeland et al., 2001). Once cells are switched to 42 °C for 15 minutes the genes are expressed from the lambda P_L promoter due to the

inactivation of the repressor. This allows for more efficient and considerable recombination (Copeland et al., 2001; L. Thomason et al., 2007).

The short induction time greatly reduces cellular stress and unwanted recombination. The phage system conveniently contains the CI-repressor, which is auto regulated and more concisely controlled as opposed to recombination involving multicopy plasmids that are expressed from heterologous promoters. Many times, heterologous promoters run the risk of being leaky which causes unnecessary expression and inadequate side effects (Sharan et al., 2009).

In the lambda prophage, the P_L operon which encodes the Red recombination genes are tightly controlled by the temperature sensitive lambda CI-repressor (Datta, Costantino, & Court, 2006; Lee et al., 2001; Liu et al., 2003; Sergueev, Yu, Austin, & Court, 2001; Yu et al., 2000). When the P_L operon is present on a plasmid, high concentrations of the CI-repressor are necessary. High concentrations of the repressor prohibit the switching on and off of the P_L operon expression. However, if the prophage is present in one copy, expression from the P_L promoter will be strong enough to promote efficient recombination without (inducing the lytic cycle of the virus) causing destruction of cell viability (Yu et al., 2000).

At temperatures as low as 30-34 °C, the CI-repressor is activated and transcription of the Red genes is shut off due to the interference with the P_L promoter. Shifting the temperature to 42 °C denatures the repressor and allows the expression of *exo*, *beta*, and *gam* functions. Shifting back to low temperatures in turn renatures the repressor which then binds to the P_L operon and once again inhibits the Red system from functioning (Datta et al., 2006; Sergueev et al., 2001).

According to Datsenko and Wanner (Datsenko & Wanner, 2000), multicopy Red plasmids and short homologies made by PCR were unsuccessful in inducing chromosomal mutations. Multicopy plasmids interfere with recombination by acting as competitive inhibitors; therefore, low copy plasmids such as pKD20 and pKD46 that encode the Red recombination system are significantly more efficient because they avoid nonessential recombination.

In previous studies, pKD20 had an optimal ribosomal binding site that allowed the Gam protein to be translated efficiently as well as express the Red function proteins from arabinose inducible promoters. Also, pKD20 contains a low copy, temperature sensitive replication origin which allows it to be eliminated effortlessly at 42°C (Abdoli et al., 2007; Datsenko & Wanner, 2000; L. Thomason et al., 2007). pKD20 was used in previous experimentation but now pKD46 is the primary plasmid utilized because it yields a notably higher amount of recombinants (Datsenko & Wanner, 2000).

P_{BAD}, an arabinose inducible promoter from the arabinose operon, controls the expression of the Exo, Beta, and Gam proteins of the lambda Red recombination system. The plasmids, pKD20 and pKD46, used to express the Red proteins, contains the gene *araC* that encodes for AraC. AraC is a regulatory protein that is transcribed under the control of the P_C promoter. When L-arabinose is present, it binds to AraC, initiating transcription of the P_{BAD} promoter and the Red proteins are expressed. When arabinose is absent, the AraC response regulator inhibits at the P_{BAD} promoter, preventing the expression of the *red* α β γ genes (Abdoli et al., 2007; Liu et al., 2003).

Shortly after the Red system was developed, Francis Stewart's lab discovered the RecET system which established that short homologies as small as 42-50 bp regions can

be included in synthetic oligonucleotides to yield successful recombination (Court et al., 2002). The RecET system was discovered as a *recB* and *recC* mutation that suppressed the *E. coli* recombination defect of the mutants. The suppression of the *recBC* (*sbcA*) mutation upregulates the expression of the recombination system from *Rac*. The RecET system is analogous to the lambda Red system in that RecE is functionally similar to Exo and RecT is functionally similar to Beta. Recombineering exploits the phage derived proteins RecE/RecT from the *Rac* prophage to assist in gene manipulation (Rivero-Müller, Lajić, & Huhtaniemi, 2007). RecET is a ssDNA binding protein that stabilizes the intermediate that anneals to the newly introduced complementary DNA strand during replication. A Gam homolog is not present in the RecET system. RecET is more efficient than the lambda Red proteins especially in linear-linear recombination interactions (Sharan et al., 2009).

1.6 Red/ET used in other organisms

Saccharomyces cerevisiae, a species of yeast, is a eukaryotic model organism studied most often in molecular biology, cellular biology, and genetics. Previous *Saccharomyces cerevisiae* studies showed that transformation using PCR fragments with selectable markers and having 35 nucleotides flanking DNA homologous to the chromosome yielded gene modifications (Datsenko & Wanner, 2000). This technique involves replacing a chromosome sequence with a selectable marker such as an antibiotic resistance gene that is produced by using PCR and generating primers containing 36 nucleotide extensions. (Datsenko & Wanner, 2000; L. Thomason et al., 2007). According to Copeland et al (2001), Agnès Baudin and associates deleted a yeast gene and replaced it with a yeast selectable marker named HIS3 using homologous recombination. PCR was

then performed to amplify HIS3 with primers 19 bp long homologous to the 3' end and 35-51 bp long at the 5' end homologous to the potential gene deletion. Once introduction of the HIS3 targeting cassette and selection for recombinants was completed, a yeast gene replacement was detected (Copeland et al., 2001). Experimentally, this provided the evidence that genes can be disrupted by transformation with PCR products containing a selectable marker and containing short homology sequences (Datsenko & Wanner, 2000; Yu et al., 2000).

lambda Red recombination was also used to delete a gene encoding lactate dehydrogenase in *Enterobacter aeogenes*. A lactate dehydrogenase-deleted mutant was first constructed to improve the productivity of 2,3-butanediol. Scientists were able to obtain 16.7% more 2,3-butanediol than the wild-type and produced a very small amount of lactate. Another study used the lambda red system to overexpress *ramA* and synonymously inactivate *acrB* with a gentamicin resistance cassette in *Enterobacter cloacae* (Hornsey et al., 2010; Jung, Ng, Song, Lee, & Oh, 2012).

CHAPTER II: HYPOTHESIS

I hypothesize that recombineering will work in *Enterobacter* sp. YSU by replacing ampicillin in pBR322 with a kanamycin or chloramphenicol resistance gene. I suspect that the lambda Red recombination system will be successful in *Enterobacter* sp. YSU because it was developed in *Escherichia coli* and has been proven to be successful in *Enterobacter cloacae*, *Enterobacter aerogenes* and *Saccaromyces cerevisiae*. (Court et al., 2002; Hornsey et al., 2010; Jung et al., 2012; Yu et al., 2000). Since pKD46 contains ampicillin resistance as a selectable marker and *Enterobacter* sp. YSU is already resistant to ampicillin, the ampicillin resistance gene in pKD46 must be replaced by the kanamycin or the chloramphenicol resistance gene. These new constructs will be transformed into *Enterobacter* sp. YSU and tested for recombineering activity.

CHAPTER III: METHODS

3.1 Growth Medium

Lennox LB was obtained from Fisher Scientific (Fair Lawn, NJ) and consisted of 10 g/L tryptone (Fisher Scientific, Fair Lawn, NJ), 5 g/L yeast extract and 5 g/L sodium chloride. When necessary, LB was supplemented with 1.6% Agar (Amresco, Inc., Solon, OH) and with 50 µg/ml kanamycin (Amresco, Inc., Solon, OH), 100 µg/ml ampicillin (Amresco, Inc., Solon, OH), or 20 µg/ml chloramphenicol (Amresco, Inc., Solon, OH). SOC medium contained 0.5% (w/v) yeast extract (Fisher Scientific, Fair Lawn, NJ), 2% (w/v) tryptone (Fisher Scientific, Fair Lawn, NJ), 10 mM sodium chloride (Fisher Scientific, Fair Lawn, NJ), 2.5 mM potassium chloride (Fisher Scientific, Fair Lawn, NJ), 10 mM magnesium chloride (Fisher Scientific, Fair Lawn, NJ), 20 mM magnesium sulfate (Fisher Scientific, Fair Lawn, NJ) and 20 mM glucose (Fisher Scientific, Fair Lawn, NJ).

3.2 Bacterial Strains

Enterobacter sp. YSU was isolated from Poplar Creek in Oak Ridge, TN (Holmes et al., 2009). *Escherichia coli* (*E. coli*) strain ECD100D *pir* and ECD100D *pir116* was purchased from Epicentre (Madison, WI).

3.3 Plasmids

Plasmids used in this experiment consisted of pKD46 provided by Dr. Nina Stourman, pACYC184 obtained from New England BioLabs Inc. (Beverly, MA), and pBR322 obtained from New England Biolabs. The kanamycin resistance gene was obtained by PCR from the EZ-Tn5 R6Kγ kan transposome (Epicentre, Madison, WI). Plasmid maps were drawn using Vector NTI Advance.

Table 1. List of primers used for PCR

PRIMERS	NUCLEOTIDE SEQUENCE
CmR Hind III	5' ACCAAGCTTCTGTTGATACCGGGAAGCC 3'
CmF Hind III	5' ACCAAGCTTAGGCGTAGCACCAGGCGT 3'
AraCF	5' TGCCACCTGCATCGATTAT 3'
OriR	5' TTTCGTTCCACTGAGCGT 3'
KanF14-H3	5' ACCAAGCTTGCATTCACAGGGTGTCTCAA 3'
KanR14-H3	5' ACCAAGCTTGCCAGTGTTACAACCAATTAACC 3'
Cm F – pBR	5'ATACATTCAAATATGTATCCGCTCATGAGACAATAAC CCTGATAAATGCTTCAATACTGTTGATACCGG 3'
Cm R-pBR	5'GTCTGACGCTCAGTGGAACGAAAACCTCACGTTAAGGG ATTTTGGTCATGAAGGCGTAGCACCAGGCGT 3'
Kan F-pBR	5'GTCTGACGCTCAGTGGAACGAAAACCTCACGTTAAGGG ATTTTGGTCATGAGCATTACAGGGTGTCTCAA 3'
Kan R-pBR	5'CAAATATGTATCCGCTCATGAGACAATAACCCTGATA AATGCTTCAATAGCCAGTGTTACAACCAATTAACC 3'

3.4 Plasmid DNA Purification

Plasmid DNA purification was carried out using Promega's (Madison, WI) Wizard® Plus SV MiniPrep DNA purification kit. 10 ml of overnight culture was harvested by centrifuging at 8,000 x g for 5 min. The supernatant was poured off and the remaining pelleted cells were resuspended with 250 µl of Cell Resuspension Solution and transferred to a 1.5 ml microcentrifuge tube. 250 µl of Cell Lysis Solution was added to each sample and inverted 4 times to mix. 10 µl of Alkaline Protease Solution was added, inverted 4 times to mix, and then incubated for 5 minutes at room temperature. 350 µl of Neutralization solution was added and inverted 4 times to mix. This was followed by 10 minutes of centrifugation at 14,000 x g. The spin column was then inserted into the collection tube and the cleared lysate was decanted into the spin column. The spin column was then centrifuged at maximum speed for 2 minutes at room temperature and the flow through was discarded. Next, 750 µl of Wash Solution containing ethanol was

added to the spin column and centrifuged at maximum speed for 1 minute. The flow through was once again discarded and the column was reinserted into the collection tube. This step was repeated with 250 µl of Wash Solution and centrifuged at maximum speed for 2 minutes at room temperature. The spin column was then transferred to a new sterile 1.5 ml microcentrifuge tube and 100 µl of Nuclease Free Water was added to the spin column. The sample was then centrifuged at maximum speed for 1 minute at room temperature and then stored at -20 °C.

3.5 Genomic DNA Purification

Genomic DNA was purified using the Wizard Genomic DNA purification kit from Promega (Madison, WI). 1 ml of overnight culture was centrifuged at 15,000 x g for 2 minutes. The supernatant was removed and the pelleted cells were resuspended in 600 µl of Nuclei Lysis Solution. The sample was incubated at 80°C for 5 minutes and then cooled to room temperature. Once cooled, 3 µl of RNase was added to the sample and inverted 2-5 times. The sample was then incubated at 37°C for 15 minutes and then cooled to room temperature. 200 µl of Protein Precipitation Solution was added and then the sample was vortexed for 20 seconds to mix. Next, the sample incubated on ice for 5 minutes and centrifuged for 3 minutes 15,000 x g. The supernatant containing the DNA was transferred to a new 1.5 ml centrifuge tube containing 600 µl of isopropanol and mixed by inverting until a thread-like precipitate appeared. The DNA sample was then centrifuged at 14,000 x g for 2 minutes to pellet the DNA, and the supernatant was poured off and allowed to fully drain upside down on a paper towel. 600 µl of 70% ethanol was added and then inverted to wash the pellet followed by centrifugation at 14,000 x g for 2 minutes. The ethanol was discarded and the pellet was allowed to air dry

for 10-15 minutes. Lastly, 100 µl of DNA Rehydration Solution was added and the sample was incubated overnight at 4 °C.

3.6 Agarose Gel Electrophoresis

A 0.8% (w/v) agarose gel was prepared by adding 1.04 g of agar (Fisher Scientific, FairLawn, NJ) to 130 ml of Tris Borate EDTA (TBE) buffer containing 0.089 tris M, 0.089 M Borate and 0.002 M EDTA (Amresco, Solon, OH). It was then heated in the microwave for about 2 minutes with frequent swirling. This was repeated until agarose was completely dissolved. It was then slightly cooled and carefully poured into a gel tray. Combs to make wells were inserted and removed once gel solidified. The gel was then placed in a gel box and submerged in 1X TBE buffer. EZ-vision dye (Amresco, Solon, OH) was mixed with the DNA samples and loaded into the wells. The gel box was covered with its lid and an electric current of 100V was applied to the gel. The gel was then run for about 30 minutes and an image of the gel was captured using the UltraCam Imaging Systems (Ultra-Lum, Inc. Claremont, CA).

3.7 DNA Digestion

14 µl of pKD46-cm purified plasmid DNA was digested using 1 µl *HindIII*-HF (New England Biolabs Inc., MA), 2 µl 10x cutsmart buffer (New England BioLabs Inc. Beverly, MA), and 3 µl of nuclease free water. The digestion was then incubated at 37°C for one hour.

14 µl of pKD46-kan purified plasmid DNA was digested using 1 µl *HindIII*-HF (New England Biolabs Inc., MA), 2 µl 10x cutsmart buffer (New England BioLabs Inc. Beverly, MA), and 3 µl of nuclease free water. The digestion was then incubated at 37°C for one hour.

5 µl of pBR322-cm purified plasmid DNA was digested using 1 µl *HindIII*-HF (New England Biolabs Inc., MA), 1 µl *PvuII*-HF (New England Biolabs Inc., MA), 1 µl 10x cutsmart buffer (New England BioLabs Inc. Beverly, MA), and 12 µl of nuclease free water. The digestion was then incubated at 37°C for one hour.

5 µl of pBR322-kan purified plasmid DNA was digested using 1 µl *HindIII*-HF (New England Biolabs Inc., MA), 1 µl *XhoI* (New England Biolabs Inc., MA), 1 µl 10x cutsmart buffer (New England BioLabs Inc. Beverly, MA), and 12 µl of nuclease free water. The digestion was then incubated at 37°C for one hour.

3.8 Polymerase Chain Reaction (PCR)

PCR reactions consisted of 12.5 µl of Q5 High-Fidelity 2X Master Mix (New England BioLabs Inc. Beverly, MA), 1.25 µl of 10 µM forward and reverse primers (Table 1), 1 µl of template DNA, and 9 µl of nuclease free water. PCR reactions were carried out in the thermal cycler using the following program: 98°C for 1 minute, 30 cycles of 98°C for 10 seconds (denaturation), 65-67°C for 1 minute (annealing), 72°C for 1 minute (extension), and hold at 10°C. Chloramphenicol resistance was amplified using the primers, CmF *HindIII* and CmR *HindIII*, the template, pACYC184, and 65°C as the annealing temperature. Kanamycin resistance was amplified using the primers, KanF14H3 and KanR14H3, the template, R6Kγ-kan transposon, and 65°C as the annealing temperature. The replication origin, *red* genes and *araC* gene (Figs 1 and 2) were amplified from pKD46 using the primers, AraCF and OriR, and an annealing temperature of 67°C. All primers were purchased from Integrated DNA Technologies (Coralville, IA).

3.9 PCR Purification

PCR purification was carried out using the QIAquick PCR purification Kit. 5 volumes of Buffer PB was added to 1 volume of PCR product and mixed. If the color of the mixture was orange or violet, 10 μ l of 3 M sodium acetate with a pH of 5 was added and mixed. The mixture should turn yellow. Next, a QIAquick spin column was placed into a 2 ml collection tube. The sample was added to the column and centrifuged for 30-60 seconds to bind the DNA. The flow through was discarded and the column was placed back in the tube. 0.75 ml of Buffer PE was added to the column to wash the sample and then centrifuged for 30-60 seconds. The flow through was once again discarded and the column was placed back in the tube and centrifuged for an additional minute. The column was placed in a clean 1.5 ml microcentrifuge tube. To elute the DNA, 50 μ l of Buffer EB (10 mM Tris-Cl, pH 8.5) was added and centrifuged for 1 minute. The sample was then separated by gel electrophoresis.

3.10 Preparation of Electroporation Competent Cells

100 ml of cells were grown with the addition of the appropriate antibiotic, if required, at 30 °C in LB medium to an O.D. (600 nm) between 0.4 and 0.6. Cells were cooled on ice in two 50 ml centrifuge tubes and kept on ice. The cells were then pelleted at 4 °C and 7,000 x g for 5 minutes in two 50 ml tubes and resuspended in 15 ml of sterile ice cold water, followed by the addition of 35 ml of ice cold sterile water. The cells were pelleted at 7,000 x g for 5 minutes at 4°C and washed again with sterile ice cold water for a total of two washes. After pouring off the supernatant, the cells were resuspended in a volume of ice cold water that was equal to the volume of the cell pellet (~150 μ l).

3.11 Preparation of Electroporation Competent Cells for Recombineering

100 ml of cells were grown with the addition of the appropriate antibiotic, if required, at 30 °C in LB medium to an O.D. (600 nm) between 0.4 and 0.6. 100 µl of 1 M arabinose was added and grown an additional 30 minutes. Cells were cooled on ice (4 °C) in two 50 ml centrifuge tubes and kept on ice. The cells were then pelleted at 4 °C and 7,000 x g for 5 minutes in two 50 ml tubes and resuspended in 15 ml of sterile ice cold water, followed by the addition of 35 ml of ice cold sterile water. The cells were pelleted at 7,000 x g for 5 minutes at 4°C and washed again with sterile ice cold water for a total of two washes. After pouring off the supernatant, the cells were resuspended in a volume of ice cold water that was equal to the volume of the cell pellet (~150 µl).

3.12 Transformation by Electroporation

40 µl of competent cells was added to 1 µl of DNA and mixed. The mixture was placed into an ice-cold electroporation cuvette (BioExpress, Kaysville, UT) with a 2 mm gap that has been stored in a -20°C freezer. The mixture was tapped to the bottom of the tube. The cells were shocked at 25 µF, 200 ohms, and 2.5 kV and then immediately mixed with 960 µl of SOC medium to prevent the cells from dying. The cells were incubated at 30 °C for 45-60 minutes by shaking and then plated using 100 µl of cells on LB agar plates containing the appropriate antibiotic and incubated overnight at 37°C.

3.13 Preparation of Chemically Competent Cells

100 ml of cells was grown at 30°C in LB medium to an O.D. (600 nm) of 1.0. Cells were cooled on ice (4°C) in two 50 ml centrifuge tubes and kept on ice. The cells were then pelleted at 4°C and 7,000 x g for 5 minutes in two 50 ml tubes and resuspended in 0.15 M NaCl. The cells were pelleted at 7,000 x g for 5 minutes at 4°C and

resuspended in transformation buffer containing, . 1 ml of ice-cold transformation buffer containing 15% glycerol, 0.1 M CaCl₂, 10 mM Tris-HCL, pH 8.0 and 10 mM MgCl₂. 400 µl of the resuspended cells were distributed to pre-chilled sterile 1.5 ml tubes and incubated overnight on ice in the refrigerator. To make the cells competent, they were frozen at -80°C.

3.14 Transformation by Heat Shock

Competent cells were obtained from the -80 °C freezer and thawed out on ice. 100 µl of cells and 1 µl of DNA was mixed in a 1.5 ml microcentrifuge tube and incubated on ice for 30 minutes. The microcentrifuge tubes containing the cells and DNA were heat shocked in a water bath at 42°C for 50 seconds. The cells were put back on ice and 900 µl of LB medium was immediately added and the mixed with the cells. The cells were then incubated at 37°C in a shaker for 45-60 minutes. 100 µl of cells were plated on LB agar containing kanamycin or chloramphenicol and incubated overnight at 37°C.

3.15 Polynucleotide Kinase Treatment and Ligation

5 µl of pKD46 plasmid, 3µl of cm or kan fragment, 1 µl 10x T4 DNA ligase buffer (New England BioLabs Inc. Beverly, MA), and 1 µl of T4 Polynucleotide Kinase (PNK) (New England BioLabs Inc. Beverly, MA) was added to 1.5 ml microcentrifuge tube and incubated at 37°C for half hour. After incubation, 1 µl of T4 DNA ligase (New England BioLabs Inc. Beverly, MA), was mixed in a 1.5 ml microcentrifuge tube and incubated overnight at 4°C.

CHAPTER IV: RESULTS

4.1 Generation of a pKD46 PCR fragment

The *araC* gene through the origin of replication of pKD46 (Fig 1) was amplified to remove the *ampR* gene from this plasmid. It was necessary to remove the *AmpR* gene because *Enterobacter* sp. YSU was already resistant to ampicillin. Therefore, replacing it with another antibiotic resistance gene, which is lacking in *Enterobacter* sp. YSU, is necessary for expressing the *red* genes in this strain. The removal of *ampR* created the pKD46 PCR fragment (Fig 2) that was used for the constructs. Gel electrophoresis was used to visualize of the correct band size for the pKD46 PCR fragment was generated. The PCR fragment of interest was about 5225 bp in size and can be observed in lanes 2, 3, and 4 of figure 3.

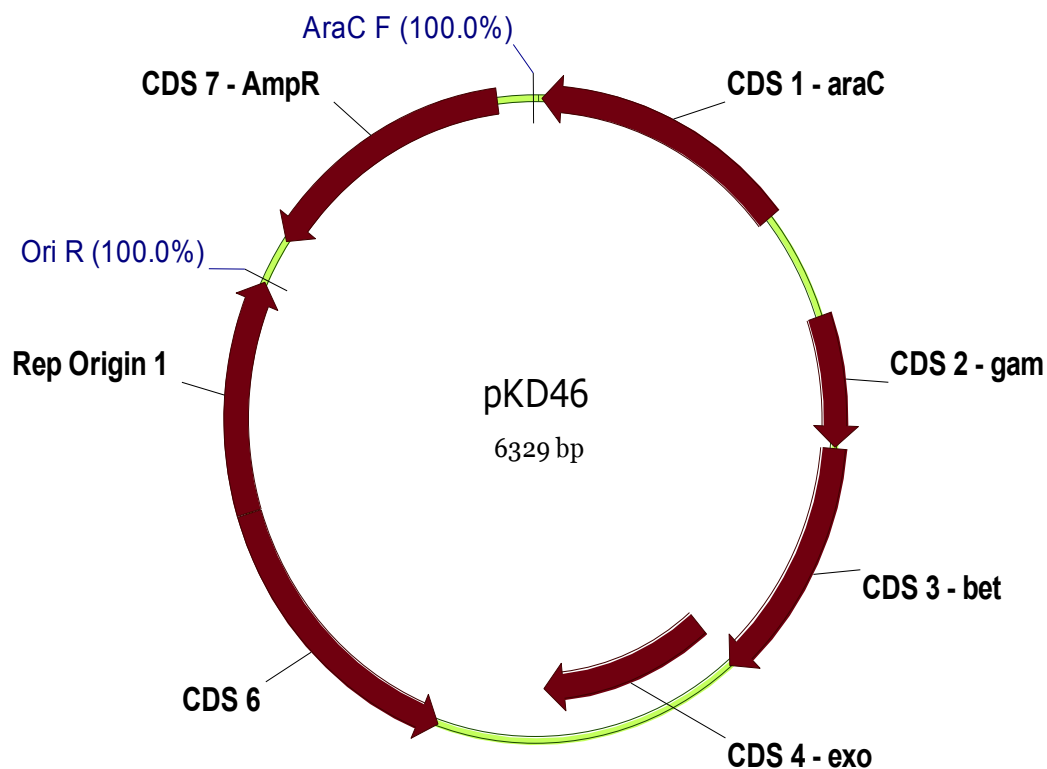


Figure 1. Map of pKD46 as drawn by Vector NTI Advance ® 11.5.0 (reference). a. A fragment containing the *araC*, *gam*, *bet*, *exo* and the replication origin were PCR amplified using pKD46 plasmid as a template and the AraC F and Ori R primers. CDS 1 – *araC* = *araC* regulator, CDS 2 – *gam* = *gam* gene, CDS – *bet* = *beta* gene, CDS4 – *exo* = *exo* gene, CDS 6/Rep Origin 1 = Replication origin, CDS 7 – *AmpR* = *ampR* gene

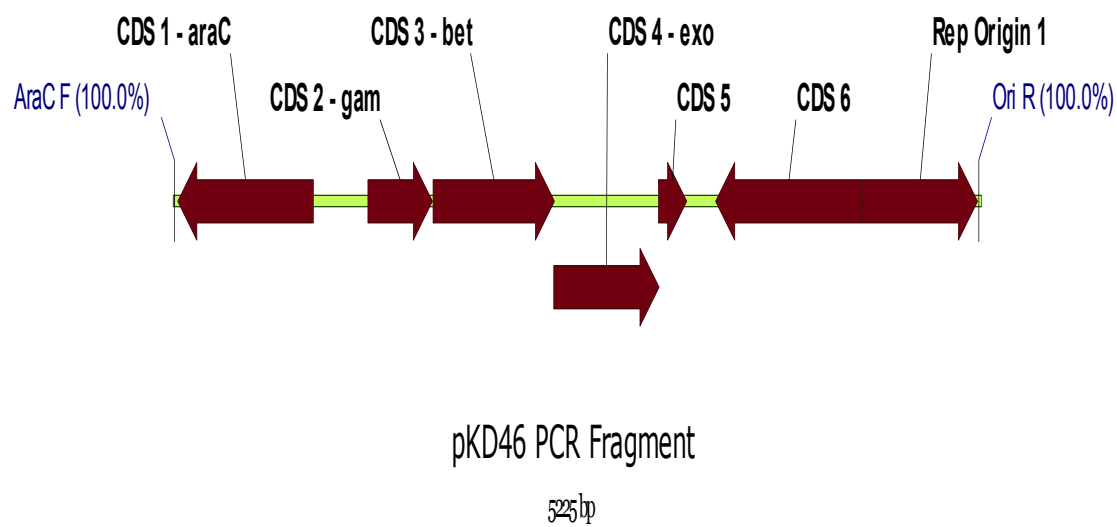


Figure 2. Map of linear pKD46 without the *ampR* gene. Genes are defined in figure 1.

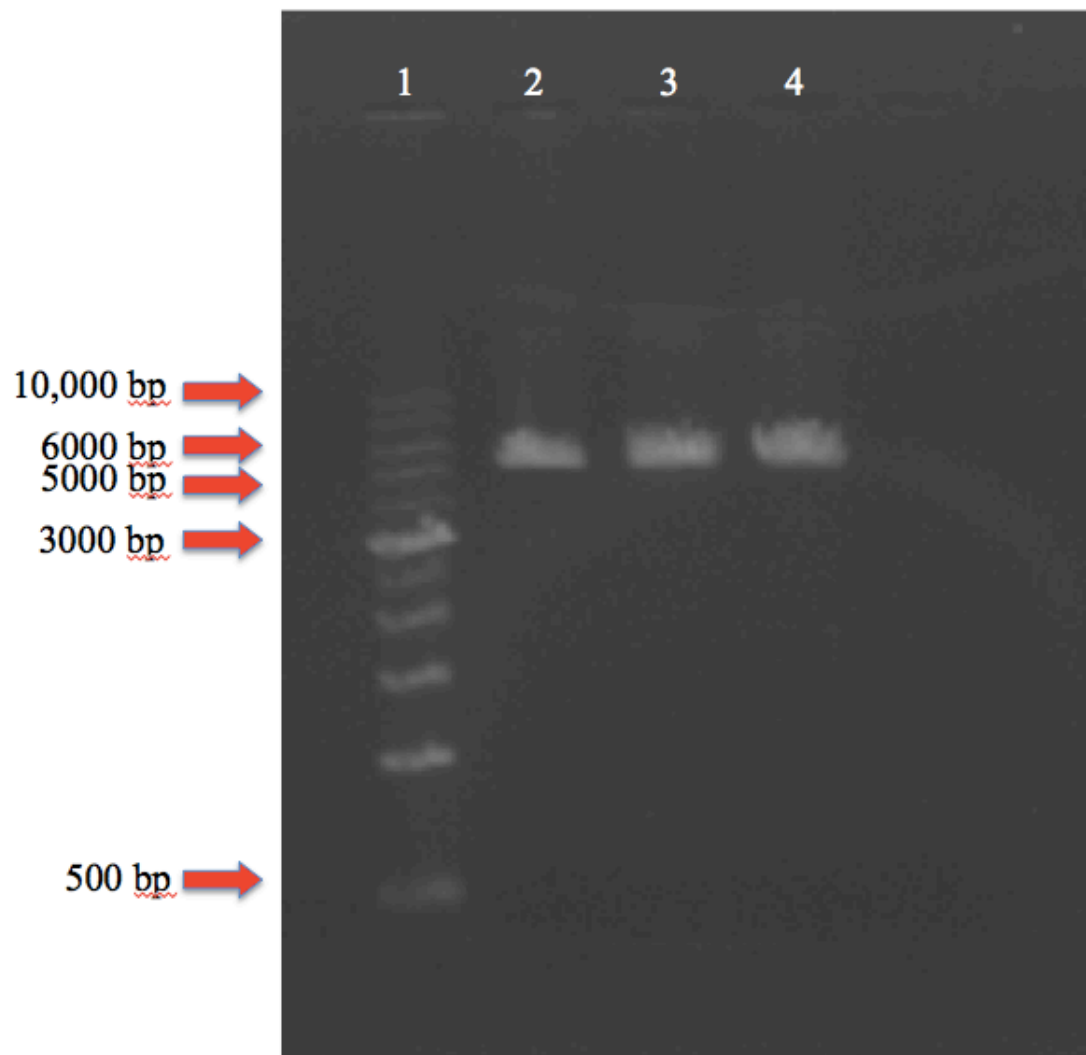


Figure 3. Gel electrophoresis of pKD46 PCR fragment. Lane 1, 1 kb ladder and Lanes 2-4, pKD46 PCR products

4.2 Generation of a *cmR* gene fragment

A *cmR* gene fragment was PCR amplified from pACYC184 (Fig 4) to be used in future the construction of a pKD46-Cm construct. Gel electrophoresis was used to visualize the band size of the *cmR* PCR fragment. In Figure 5, bands about 841 bp in size can be seen in lanes 1, 2, 3, 4, 5, and 6 with lane 7 representing the 1 kb ladder.

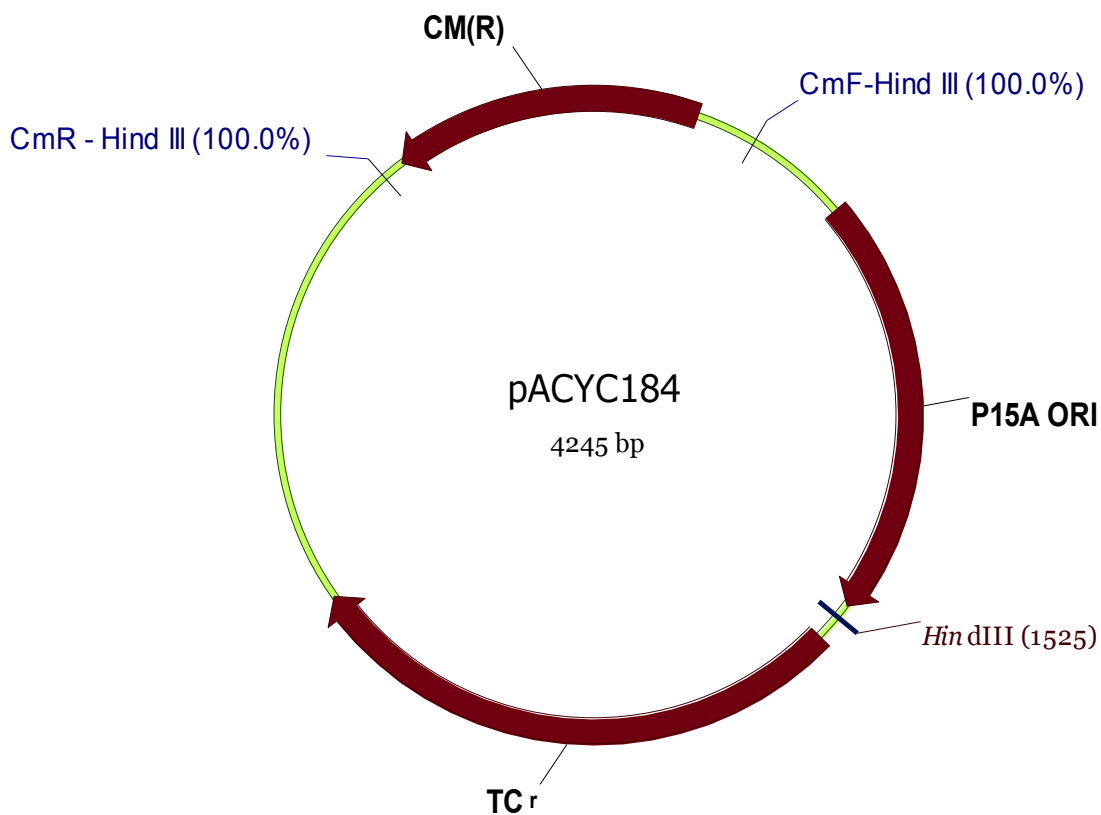


Figure 4. Vector NTI Advance ® 11.5.0 software was used to construct a pACY184 plasmid map. The *cmR* gene was PCR amplified out of the pACYC184 plasmid using CmF-*Hind*III and CmR-*Hind*III primers. CM(R) = Chloramphenicol resistance, TC^r = Tetracycline resistance, p15A Ori = Replication origin.

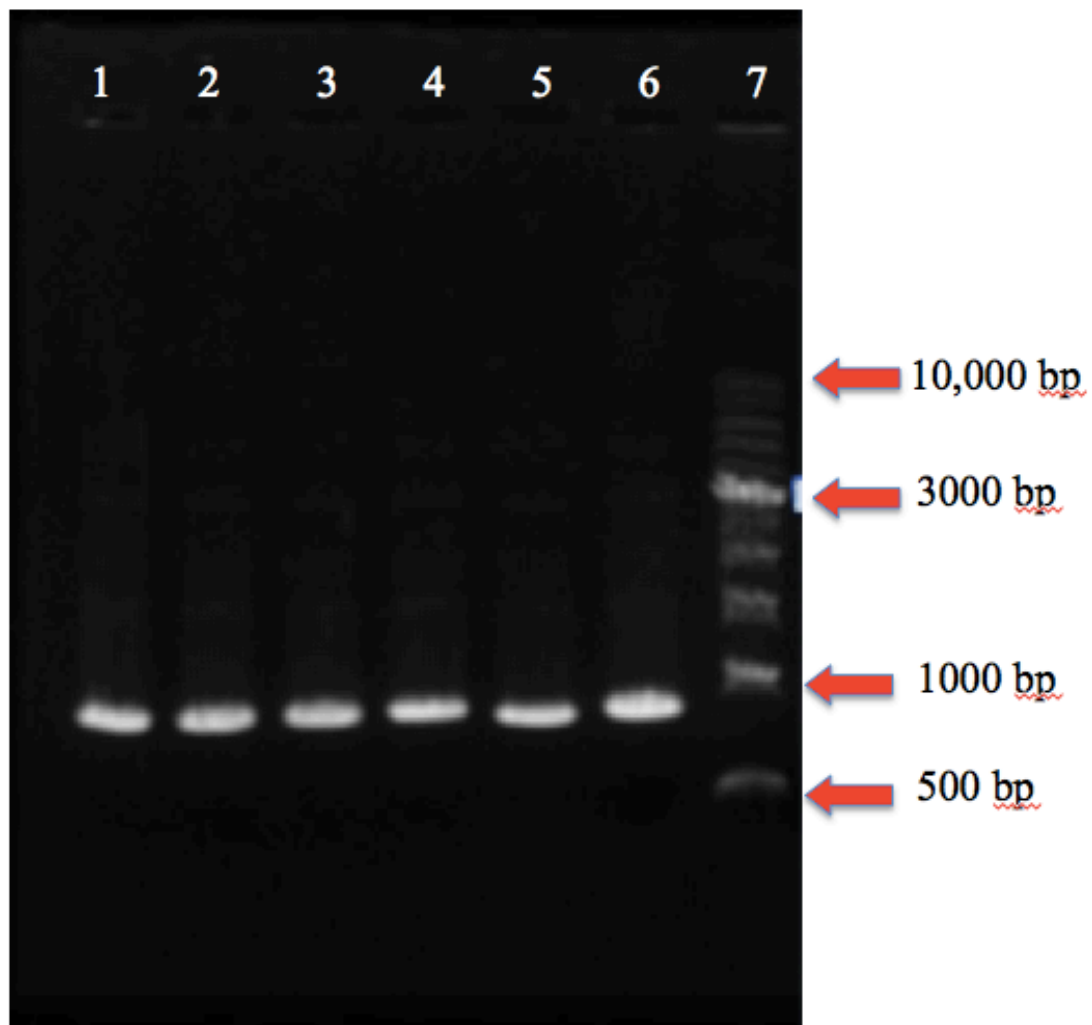


Figure 5. Gel electrophoresis of *cmR* gene PCR products. Lane 1-6, - *cmR* gene PCR products and Lane 7, 1 kb ladder

4.3 pKD46-Cm Construction

pKD46 and *cmR* fragments were purified using the Qiagen QiaQuick PCR Purification kit. The fragments were then mixed together and treated with T4 polynucleotide kinase to ligate them. The ligated DNA was then transformed into ECD100D pir116 *E. coli* and plated on LB-Cm plates. pKD46-Cm (Fig 6) transformants were then purified using Promega's Miniprep DNA Purification kit.

In Figure 7, lane 1 represents the 1 kb ladder. The uncut DNA is located in lane 2 and is approximately 6000 kb in size. The digested plasmid is located in lane 3 and is represented by a band that is approximately 5225 bp and 841 bp in size. The 5225 bp fragment represents the pKD46 piece of DNA and the 841 bp fragment represents the *cmR* fragment of DNA.

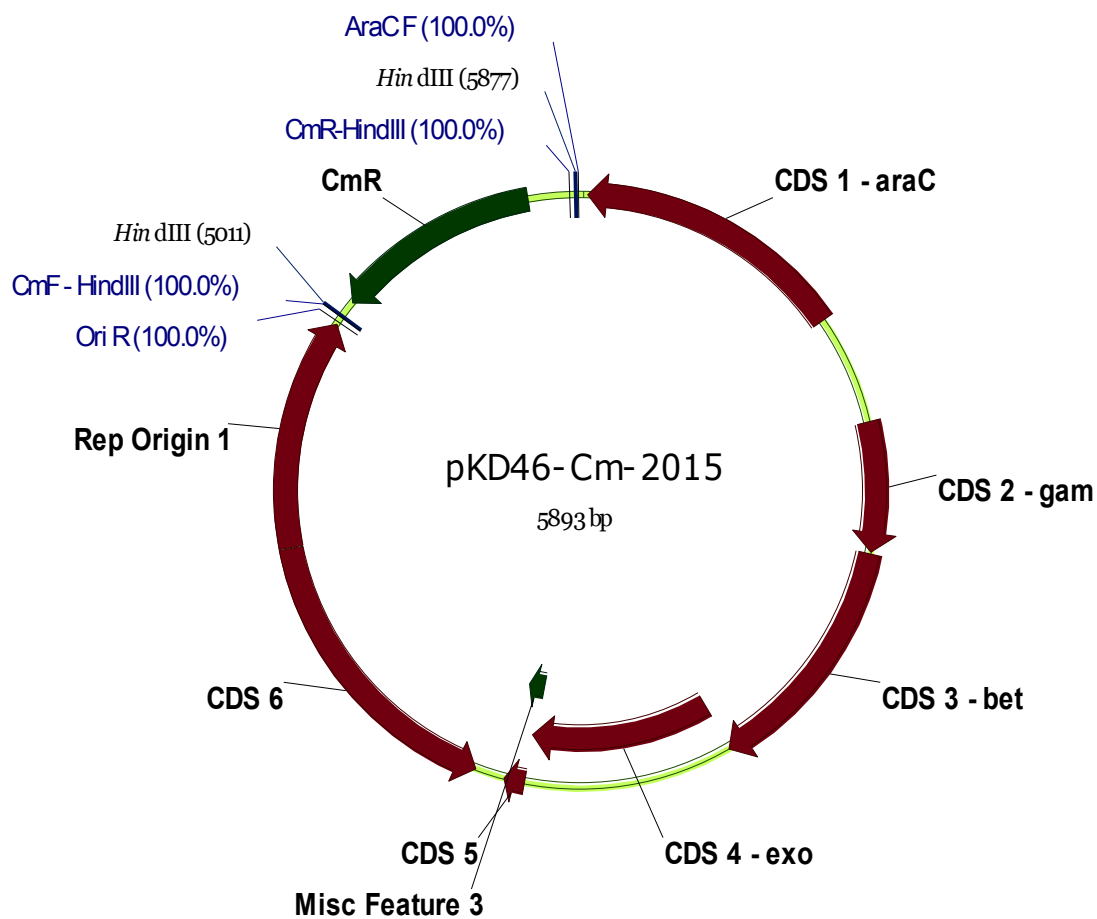


Figure 6. Vector NTI Advance ® 11.5.0 software was used to construct a pKD46–Cm plasmid map that represents the ligated pKD46-Cm plasmid construct that was used for recombination.

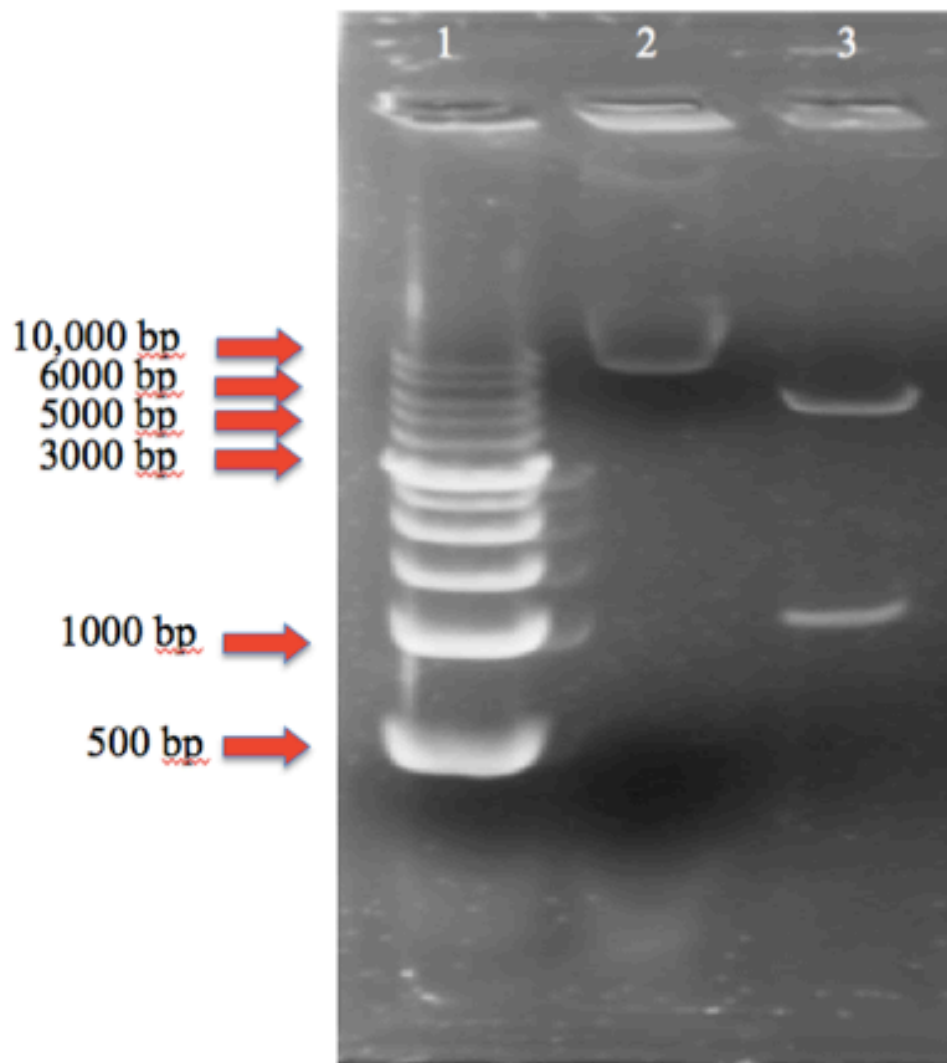


Figure 7. Gel electrophoresis of ligated pKD46-Cm plasmid. Lane 1, 1 kb ladder, Lane 2, undigested pKD46-Cm, Lane 3- *HindIII* digested pKD46-Cm.

4.4 Generation of a *kanR* gene fragment

A *kanR* gene was PCR amplified from an EZ-Tn5 transposon (Fig 8) and was used to generate the pKD46-Kan construct used for recombination. To verify the correct size of the *kanR* fragment, gel electrophoresis was applied. Lane 1 represents a 1 kb ladder and lanes 2, 3, 4, 5, 6, and 7 represent the *kanR* fragment. The fragment of interest was approximately 934 bp in size and can be visualized in Figure 9.

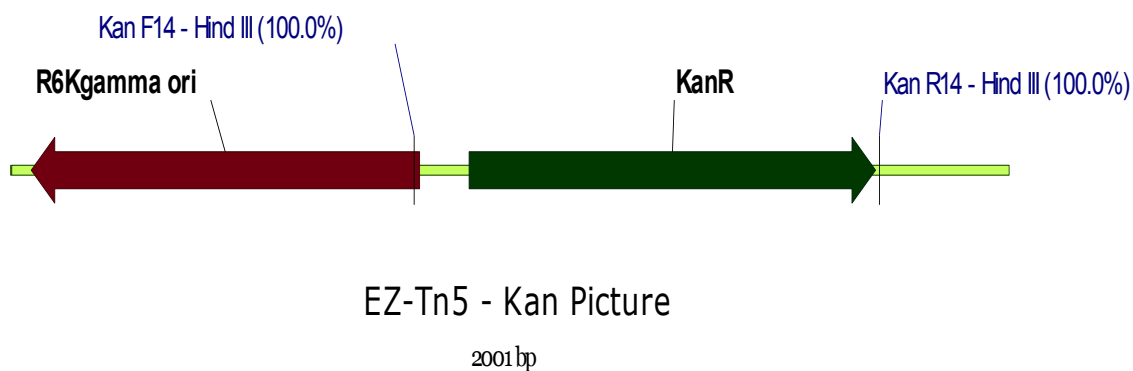


Figure 8. Vector NTI Advance ® 11.5.0 software was used to construct a linear EZ-Tn5 map representing PCR amplification of *kanR* gene from EZ-Tn5 using Kan F14-*HindIII* and Kan R14-*HindIII*. R6Kgamma ori = Replication origin, KanR = kanamycin resistance gene

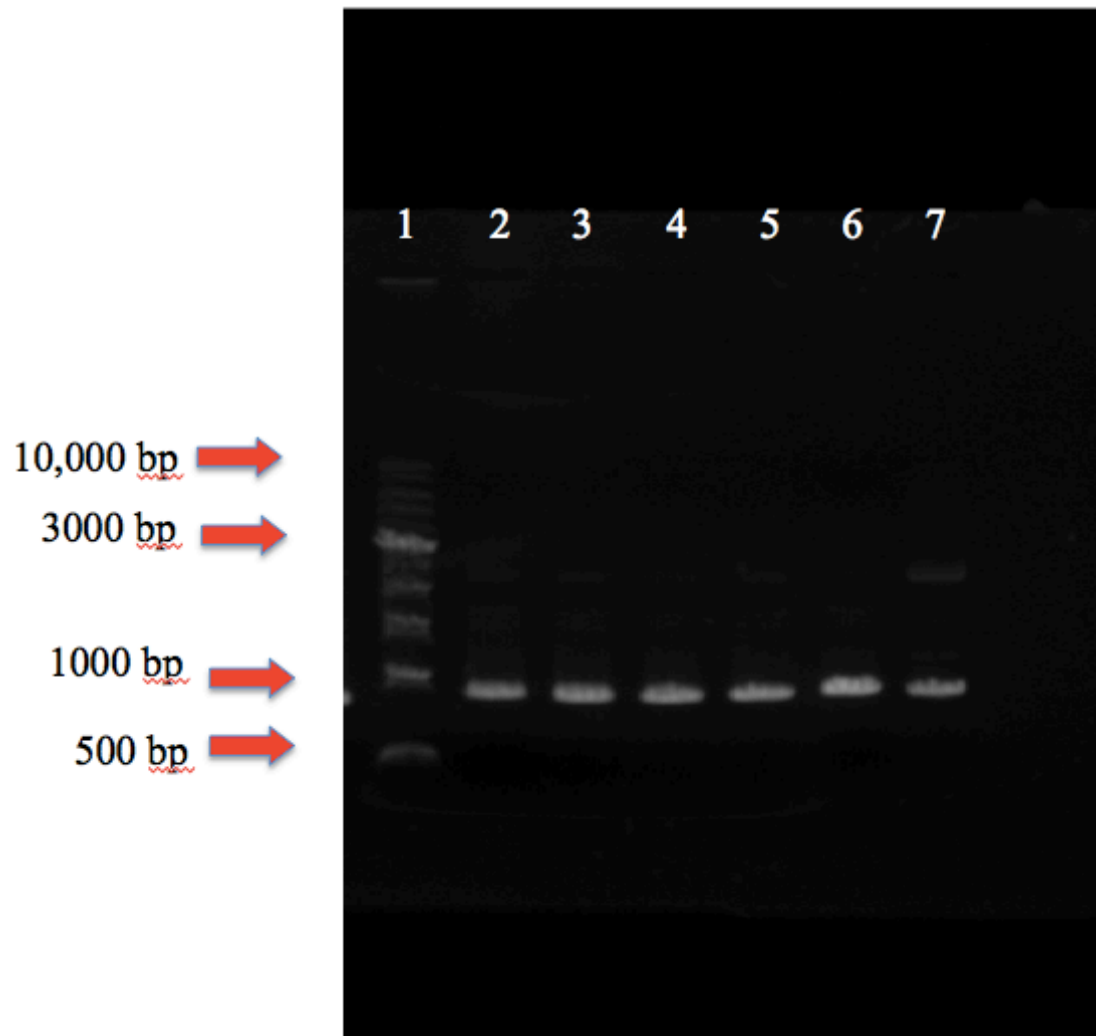


Figure 9. Gel electrophoresis of *kanR* gene PCR products. Lane 1, 1 kb ladder, Lane 2-7, *kanR* gene PCR products

4.5 pKD46-Kan Construction

pKD46 and *kanR* fragments were purified using the Qiagen QiaQuick PCR Purification kit. The fragments were then mixed together and treated with T4 polynucleotide kinase to ligate them. The ligation was then transformed into ECD100D *pir116 E. coli* and plated on LB-Kan plates. pKD46-Kan (Fig 10) transformants were then purified using Promega's Miniprep DNA Purification kit.

In Figure 11, lane 1 represents the 1 kb ladder and lanes 3, 5, and 7 are uncut pieces of DNA. Lanes 4, 6, and 8 were digested and fragments sizes are roughly 5225 bp and 941 bp. The 5225 bp fragment represents the pKD46 piece of DNA and the 941 bp fragment is the *kanR* gene.

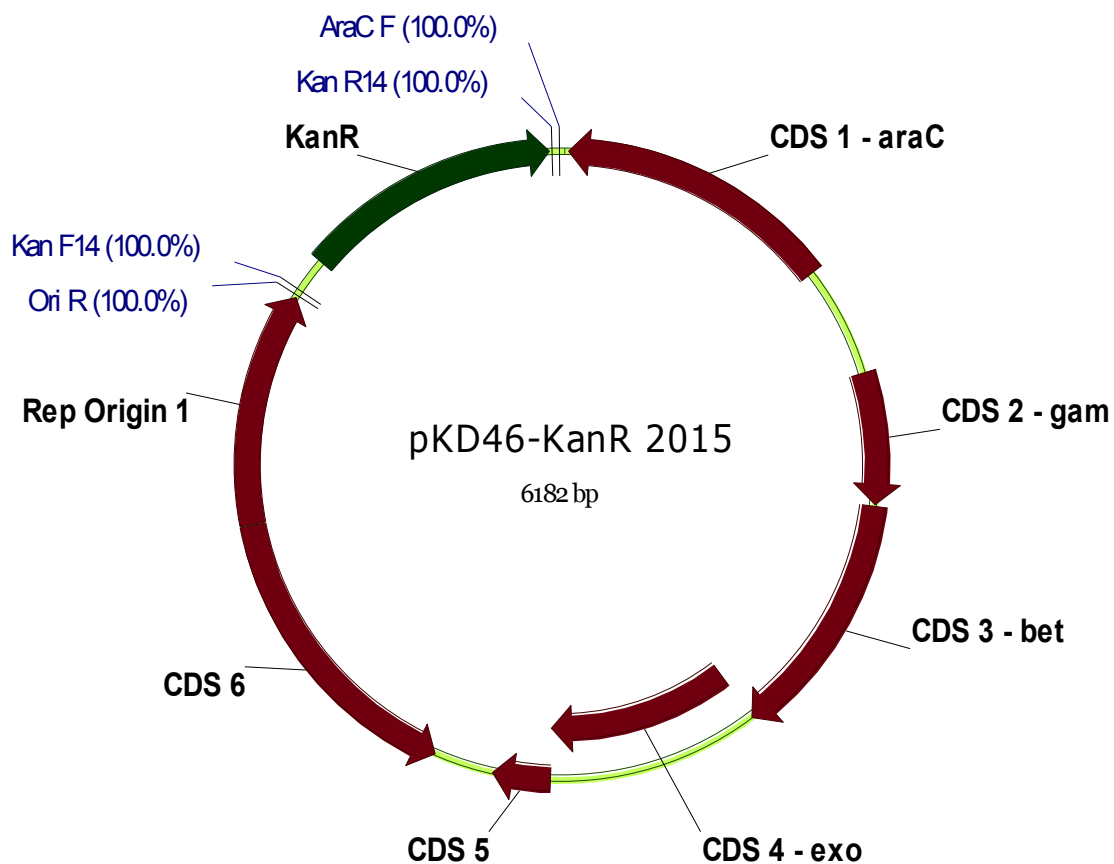


Figure 10. Vector NTI Advance ® 11.5.0 software was used to construct a pKD46 –Kan plasmid map that represents the ligated pKD46-Kan plasmid construct that will be used for recombination.

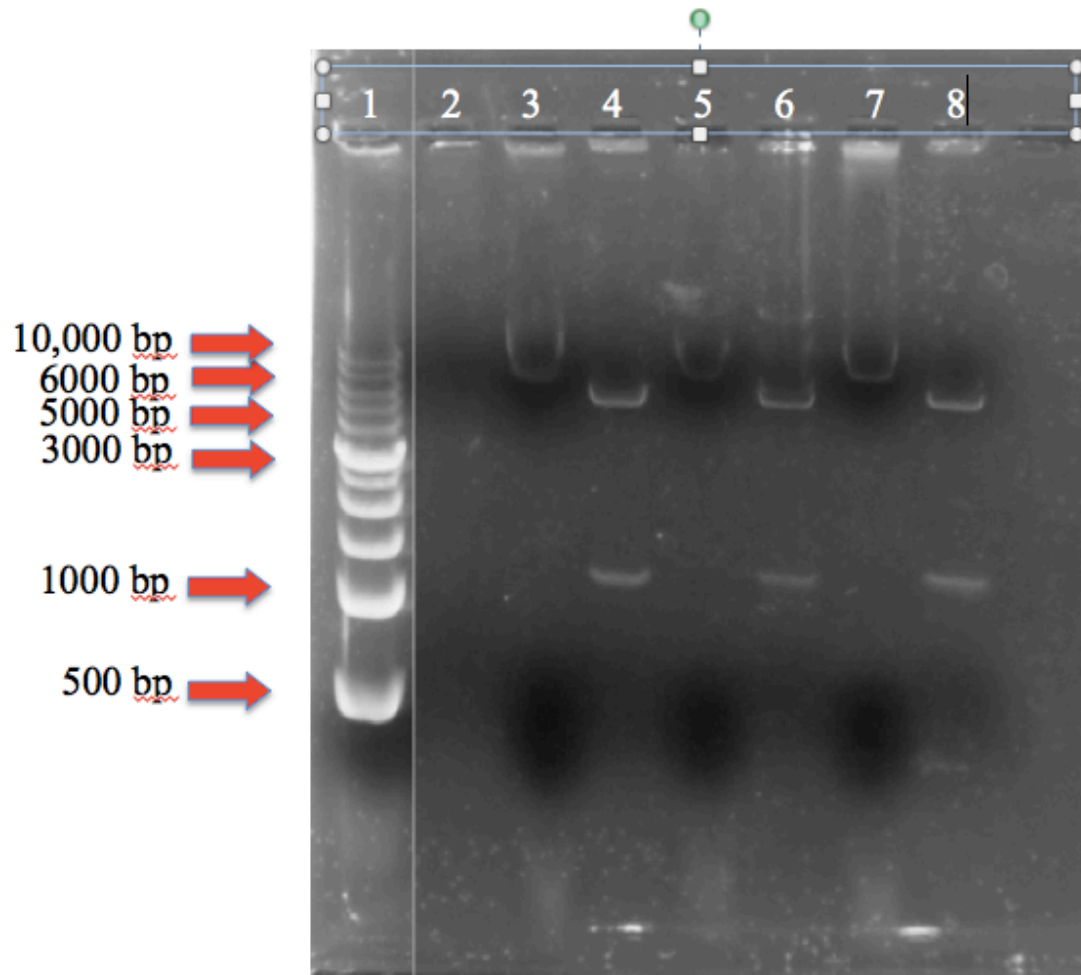


Figure 11. Gel electrophoresis of ligated pKD46-Kan plasmid. Lane 1, 1 kb ladder,, Lane 3, undigested pKD46-Kan, Lane 4, *HindIII* digested pKD46-Kan, Lane 5, undigested pKD46-Kan, Lane 6, *HindIII* digested pKD46-Kan, Lane 7, undigested pKD46-Kan, and Lane 8, *HindIII* digested pKD46-Kan

4.6 Preparation of the *cmR* and *kanR* DNA Fragments

First the *cmR* and *kanR* genes were PCR amplified from pACYC184 and the *EZ-Tn5 transposon* using the primers; Cm F- pBR, Cm R- pBR, Kan F- pBR, and Kan R- pBR because they contain homologous arms for recombination which will be used for *ampR* replacement in pBR322. The PCR products were then run using gel electrophoresis. The *kanR* fragment can be visualized in lanes 3 and 4 in figure 12 with a band size of 934 bp. The *cmR* fragment is represented by the 841 bp band present in lanes 6 and 7 of figure 12. The PCR products were then gel purified and run using gel electrophoresis, which can be seen in figure 13. Lane 2 contains the 841 bp gel purified fragment and lane 3 contains the 934 bp gel purified fragment.

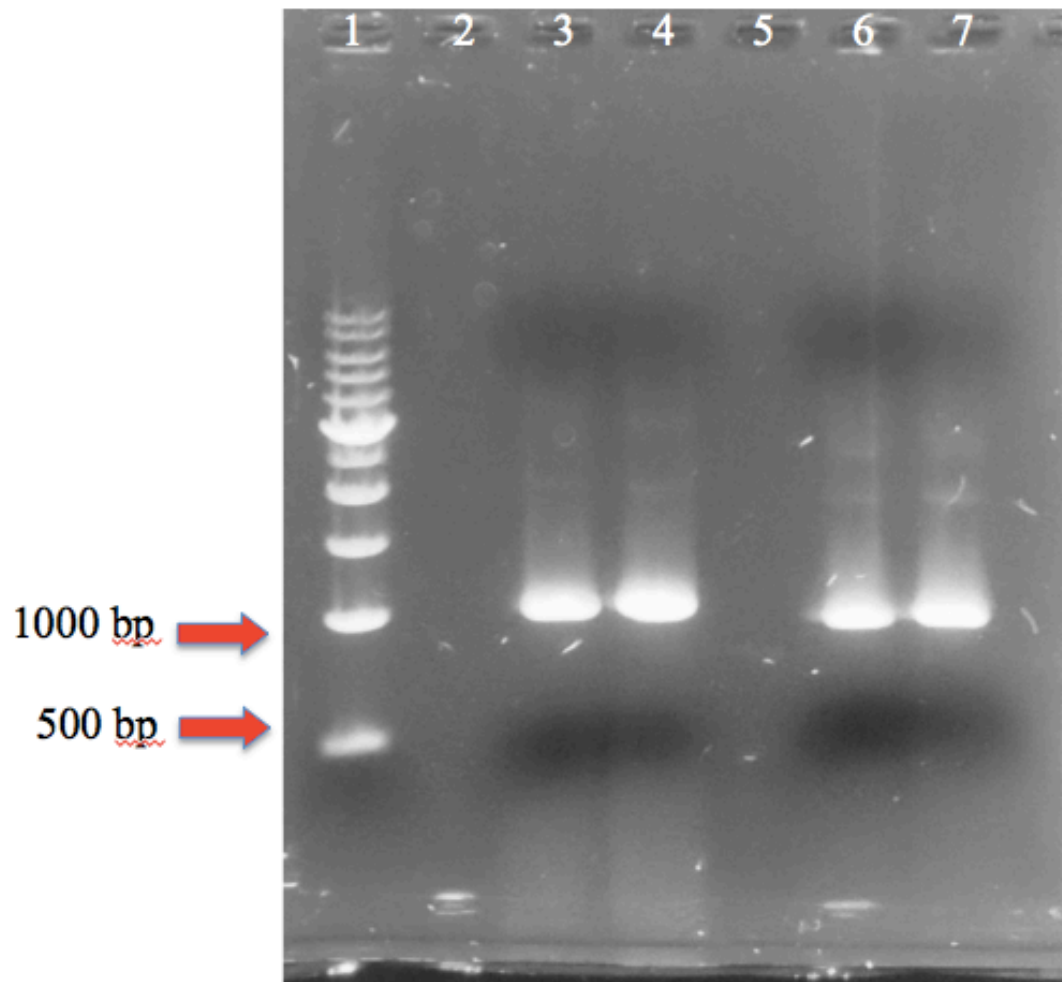


Figure 12. PCR of kanamycin and chloramphenicol resistance fragment. Lane 1, 1 kb ladder, Lane 2 is empty, Lanes 3 and 4, kanamycin PCR fragment, Lane 5 is empty, Lanes 6 and 7, chloramphenicol resistance PCR fragment

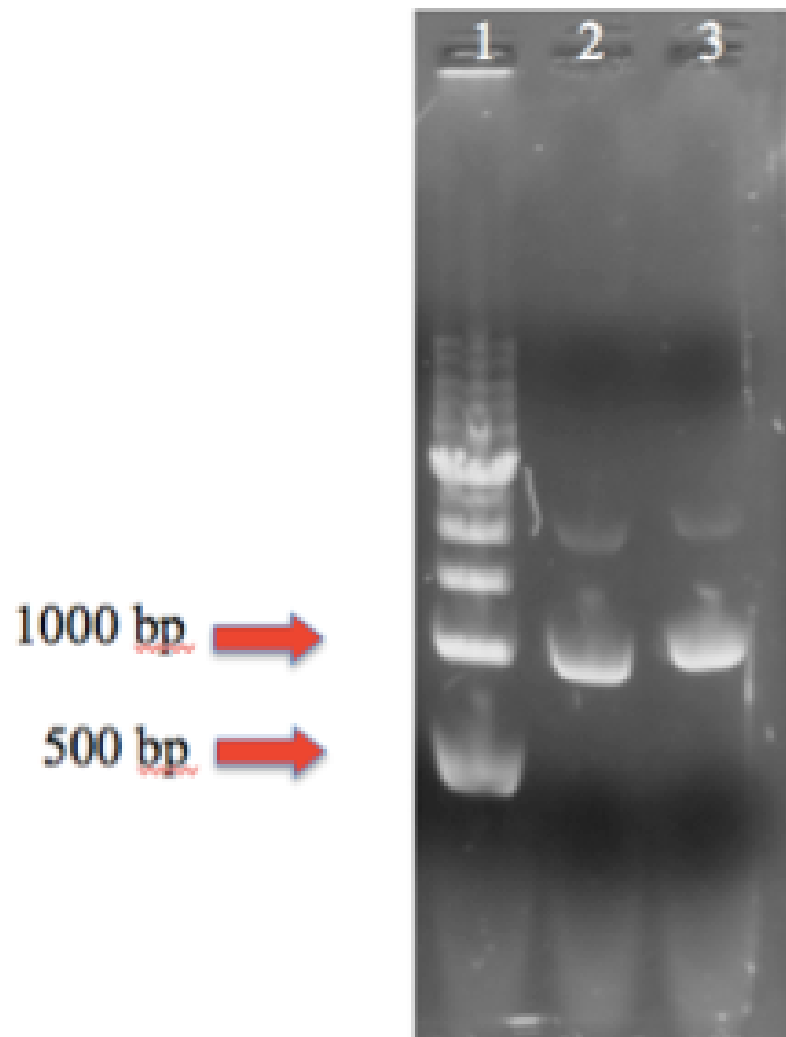


Figure 13. Gel purification of kanamycin and chloramphenicol resistance fragment. Lane 1, 1 kb ladder, Lane 2, gel purification of chloramphenicol resistance fragment, Lane 3, gel purification of kanamycin resistance fragment

4.7 Purified pBR322

pBR322 was purified and run using gel electrophoresis. Lane 1 contains the 1 kb ladder and lanes 3 and 4 represent the purified, intact pBR322 plasmid. The pBR322 plasmid was then electroporated with the kanamycin and chloramphenicol resistance fragments.

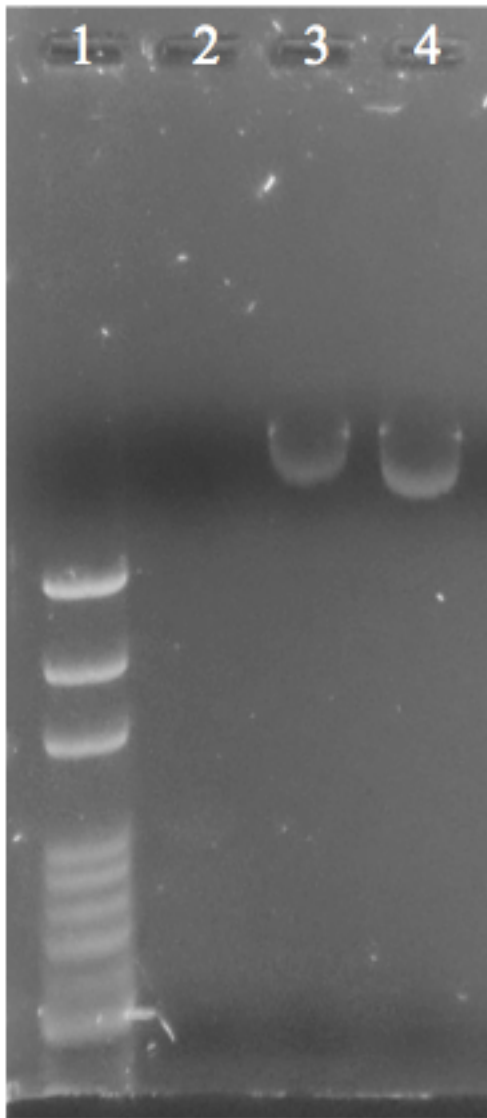


Figure 14. Purified pBR322 plasmid. Lane 1, 1 kb ladder, Lane 2 is empty, Lanes 3 and 4, purified pBR322 plasmid.

4.8 Recombineering Experiment in *E. coli* using pKD46-cm and pKD46-kan

ECD100D *pir116* (pKD46-Cm) was transformed with a mixture of pBR322 and the kanamycin resistance fragment and ECD100D *pir116* (pKD46-Kan) was transformed with a mixture of pBR322 and the chloramphenicol resistance fragment. This was done using two separate transformants for each of the kanamycin and chloramphenicol mixtures. This was done with the hopes that at least one out of the two transformants for each antibiotic resistance gene would be successful. pBR322-cm sample 1 contained 11 colonies and sample 2 contained 1 colony. pBR322-kan had 732 colonies from sample 1 and 764 colonies from sample 2. Two colonies of each were grown in overnight culture and plasmid preps were performed on the pelleted cells.

4.9 Digestion of pBR322-cm and pBR322-kan

Figure 12 represents the *HindIII* and *PvuII* digestion of pBR322-cm and *HindIII* and *XhoI* digestion of pBR322-kan. Undigested pBR322-cm plasmids are located in lanes 1 and 3 and should be 1732, 2041, and 448 bp in size. Lanes 2 and 4 are the digested pBR322-kan plasmids and should be 3315 and 1003 bp in size. The bands present in the digested pBR322-cm lanes do not appear to be the correct band size consistent with the digested fragments. Lanes 6 and 8 represent the undigested pBR322-kan plasmids. Lanes 7 and 9 are the digested pBR322-kan plasmids and should be consistent with the 3315 and 1003 bp in size representing the digested fragments. These bands appear to be the correct size. Note that the use of gel green to make the agarose gel causes the DNA bands to migrate higher than expected.

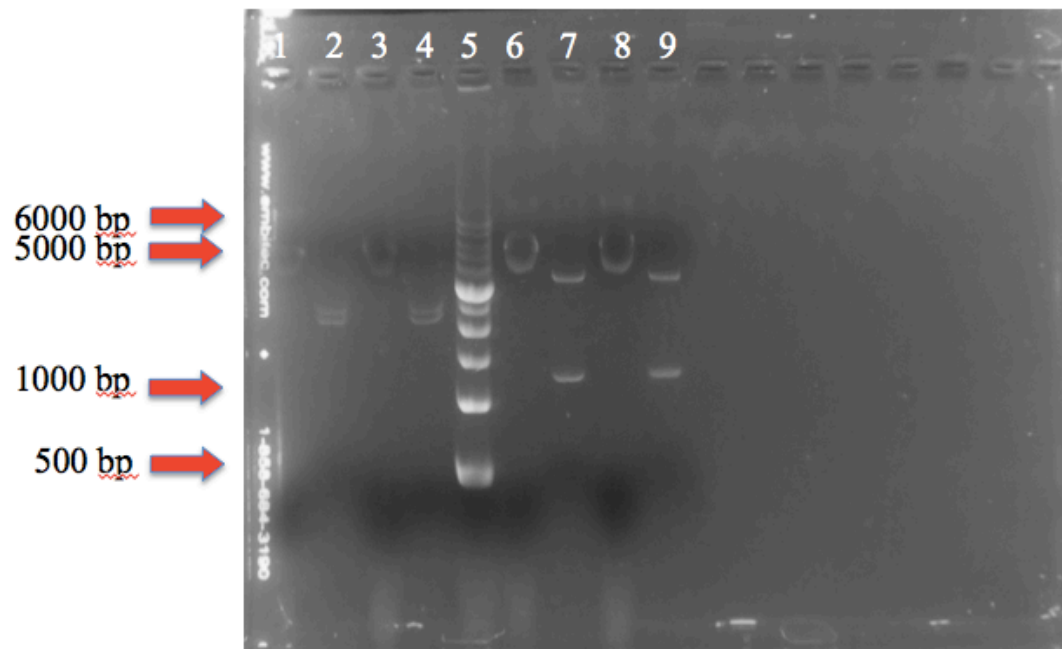


Figure 15. Gel electrophoresis of digested and undigested pBR322-Cm and pBR322-Kan. Lane 1, undigested pBR322-cm 1, Lane 2, digested pBR322-cm 1, Lane 3, undigested pBR322-cm 2, Lane 4, digested pBR322- cm 2, Lane 5, 1 kb ladder, Lane 6, undigested pBR322-kan 1, Lane 7, digested pBR322-kan 1, Lane 8, undigested pBR322-kan 2, and Lane 9, pBR322 – kan 2.

4.10 Verification of the insertion of pBR322-kan into the *ampR* region of *E. coli*

To verify that pBR322-kan was inserted into the *ampR* region of *E. coli* and also that pBR322 was present, the pBR322-kan 1 & 2 transformants were streaked for isolation on kanamycin, ampicillin, and tetracycline plates. If the pBR322-kan gene is inserted in place of the *ampR* gene then growth should be present on the kanamycin plate but not the ampicillin plate. Also, to verify that it is pBR322 plasmid that was inserted, tetracycline plates were used because pBR322 contains a *tetR* gene therefore it should grow on the tetracycline plates. After incubating for 24 hours, the plates were analyzed and were consistent with our initial predictions. There was growth present for both pBR322-kan 1 & 2 on both kanamycin and tetracycline plates but not on the ampicillin plates. Therefore, pBR322-kan was successfully inserted into the *ampR* gene and transformed into *E. coli*.

4.11 Electroporation and Transformation of pBR322-kan and pBR322-cm into *Enterobacter* sp. YSU

Since recombination was successful in *E. coli*, I attempted to transform pBR322-kan and pBR322-cm into *Enterobacter* sp. YSU. Transformation by electroporation with pKD46-kan was successful and had 33 colonies in 100µl of sample and 205 colonies in the rest of the sample. pBR322-cm, however, appeared to be unstable in *Enterobacter* sp. YSU because it took several days for colonies to appear. Thus, chloramphenicol resistance from pACYC184 does not appear to express well in YSU. This makes it difficult to attempt the pBR322 *ampR* gene replacement in YSU and therefore, may work better using a different antibiotic resistance gene.

CHAPTER V: DISCUSSION

5.1 Hypothesis Overview

After reviewing the results from all experiments, I am unable to support or refute my hypothesis that recombineering will be successful in *Enterobacter* sp. YSU by replacing an ampicillin resistance gene in pBR322 with a chloramphenicol or kanamycin resistance gene. Although I was able to successfully replace *ampR* in pBR322 with kanamycin in *E. coli*, I was unable to do so in *Enterobacter* sp. YSU because chloramphenicol resistance from pACYC184 does not express well in *Enterobacter* sp. YSU. Future research using the application of recombineering may result in higher success rates if attempting to do so using a different antibiotic resistance gene such as *tetR*. It may also be more efficient to use transposon mutagenesis to eliminate *ampR* in YSU and repeat the experiment using unmodified pKD46. Another way to avoid issues with expression is by putting genes such as *sacB*, *kanR*, and the red genes on a transposon and then trying to introduce it into YSU. Recombineering would then be able to be used to eliminate the *kanR* and *sacB* genes.

5.2 Other Uses for lambda Red

A selectable marker is a gene added to a vector that allows for easy selection of cells that contain that specific trait. Antibiotic resistance genes are commonly used as the selectable marker of choice (Copeland et al., 2001; Court et al., 2002; Lee et al., 2001; Yu et al., 2000). Genes on a BAC or plasmid can be substituted with drug resistance selectable markers by using homologous recombination (L. Thomason et al., 2007; Yu et al., 2000).

Antibiotic cassettes containing antibiotics such as chloramphenicol or ampicillin with specific flanking homology can replace specific genes on a chromosome. These replacements are selectable for drug resistance. The areas between the homology arms and resistance cassette determine the replaced/deleted region. Cassettes have been successfully inserted without deleting any bases and can also replace as much as 70 kb (Court et al., 2002; L. Thomason et al., 2007).

Selectable markers, as well as other DNA coding sequences such as *lacZ* fusions, GFP fusions, and His-tags, can be interrupted in the same region while the resistance cassette is added directly to it (Court et al., 2002).

Aside from creating selectable markers, it is also possible to create recombinants that use the *sacB* genes to remove drug resistance selectable markers through the process of counter selection (L. Thomason et al., 2007; Yu et al., 2000).

According to Yu et al. (2000), in previous experiments, genes and cassettes were fused together to encode specific tags. Tags can be inserted into a specific location on a gene to be modified involving a dual strategy. Firstly, an unselected and selectable drug marker are joined and recombined into the drug resistance selected location (Copeland et al., 2001; Yu et al., 2000). To delete a drug cassette a second round of recombination can be utilized (Court et al., 2002). The recombined cassette is then substituted using a counter selection marker. A counter selection marker and drug resistance marker must first be recombined for the second step to work efficiently.

This strategy allows any DNA segment to be cloned into a chromosome or plasmid (Copeland et al., 2001; Yu et al., 2000) and will not leave a scar behind when deleting a drug cassette. Insertion of non-selectable markers such as fusion genes, tags,

or point mutations at the recombination site can also be used with counter selection (Court et al., 2002).

Antibiotic resistant cassettes with appropriate homologies are used to target specific genes for replacement or modification on a chromosome. Targets for recombinants that are site specific such as *loxP* or *frt* can be added onto the flanked homologies of the cassette, which will allow Cre and FLP expression to remove the cassette. *SacB* is a counter selection gene that converts sucrose to a toxic form that kills *E. coli* and can be coupled with a selectable drug marker. A second round of recombination can be applied to delete the drug cassette and *sacB* by plating it on agar comprised of sucrose to select for recombinants. Counter selection allows for the deletion of the cassette and *sacB* without inducing a permanent scar. Therefore, a targeted region can be replaced by a drug cassette and *sacB* in the first step and mutant DNA can be introduced in the second step allowing selection of site specific mutations in a particular gene of interest (Copeland et al., 2001; Court et al., 2002).

A two-step procedure for BAC targeting, which allows mutations to be inserted into BACs without leaving drug resistance markers behind, has also been utilized. In the first step, PCR generated targeting cassettes combined with a fusion gene are targeted to a BAC. Cells that have taken up the cassette, which has been targeted to the BAC are selected by drug resistance and transformed with a second target to that specific region. The second cassette replaces the whole cassette and contains short sequences carrying a mutation. Recombination coupled with selection/counter selection provides a mean to generating genetic modifications such as insertions, deletions, and point mutations (Copeland et al., 2001).

Linear DNA can be targeted to a plasmid either resident in the cell or co-electroporated with linear DNA. This requires the plasmid DNA to be retransformed to produce pure recombination colonies. Therefore, a selectable marker combined with a linear fragment is useful (Court et al., 2002).

5.3 Flp/FRT

Flp/FRT recombination is dependent on specific sites of strand exchange via limited amounts of sequence homology (Copeland et al., 2001). This site specific recombination is usually done *in vivo* and requires short FRT sites called flippase recognition target sites (Copeland et al., 2001; Datsenko & Wanner, 2000). In yeast, the yeast plasmid encodes FLP, which inverts two specific regions of the plasmid, thus amplifying its copy number. The plasmid encoded Flp protein is the only necessary protein requires for inversion to take place. The Flp protein is also responsible for promoting inversions, deletions, and other intermolecular modifications (Gates & Cox, 1988).

FRT has a 34 bp sequence that allows Flp to bind to both 13 bp flanking arms by 8 bp spacers in reverse orientation. This is the region of crossover. FRT mediates cleavage ahead of the 8 bp location on the top strand and behind the bottom strand sequence. Recombination can thus only occur between two identical FRT sites (Gates & Cox, 1988).

5.4 CreLox

CreLox recombination is also site specific and is used for promoting modifications such as deletions, insertions, translocations, and inversions. Cre

recombinase is an enzyme that recombines short target sites called Lox sites. Cre and Lox are derived from bacteriophage P1 and works analogously to Flp/FRT recombination. Implementing Lox sequences into appropriate locations allow genes to be activated, repressed, or exchanged for other genes (Copeland et al., 2001; Court et al., 2002; Lee et al., 2001; Liu et al., 2003).

5.5 The Use of CRISPR for Genome Manipulation/Editing

CRISPR also known as Clustered, Regularly Spaced, Short, Palindromic Repeats is a newer genetic engineering/genome editing technique that focuses on RNA-guided DNA. It acts as an RNA-based bacterial acquired immune system by recognizing and eliminating foreign DNA. Located in the genome of the bacteria are interspaced repeated DNA sequences. In between the DNA sequences are sequences derived from viruses. The *Cas* genes are located near the CRISPR sequences and encode for proteins that have homology to DNA repair enzymes. Cas9 is derived from *Streptococcus pyogenes*, the bacteria known to produce strep infection in children and some adults. It is a protein required for the use of the CRISPR system and acts as an RNA-guided DNA endonuclease, which cleaves DNA at specific sites.

The way that CRISPR works is that first a virus invades a cell. The host cell gets a hold of the virus's genetic material and inserts in between CRISPR sequences. The host then makes RNA, which contain sequences that match the sequence of the virus, referred to primarily as the guide RNA (gRNA). The gRNA forms a complex with the protein Cas9. gRNA moves along the DNA to find the target which matches the sequence in the viral genome and binds to its complementary sequence. Cas9 then cleaves the DNA preventing the virus from being able to replicate.

One major benefit of using the CRISPR/Cas9 system is that it can be used in all model organisms. It can be engineered to target not only viral DNA but also any DNA sequence specific to the location of interest. Cas9 can recognize sequences up to 20 bp and can be tailored to specific genes. CRISPR can be used not only to manipulate or edit genomes but also to analyze gene expression in model organisms. The CRISPR/Cas9 system can be useful in treating genetic diseases, fighting infections, and increasing crop yields (Lin, Ewen-Campen, Ni, Housden, & Perrimon, 2015).

CHAPTER VII: REFERENCES

1. Abdoli, R., Amirthalingam, S., Lillquist, A., & Nutt, J. (2007). Effect of L-arabinose on the specific homologous recombination efficiency using the Lambda Red Recombinase system for gene disruption of *lacI* in *Escherichia coli* C29 cells. *Journal of Experimental Microbiology and Immunology*, 11(April), 120–124.
- Berg, P., & Singer, M. F. (1995). The recombinant DNA controversy: twenty years later. *Proceedings of the National Academy of Sciences of the United States of America*, 92(20), 9011–9013. doi:10.1073/pnas.92.20.9011
- Boyle, N. R., Reynolds, T. S., Evans, R., Lynch, M., & Gill, R. T. (2013). Recombineering to homogeneity: Extension of multiplex recombineering to large-scale genome editing. *Biotechnology Journal*, 8(5), 515–522. doi:10.1002/biot.201200237
- Chan, W., Costantino, N., Li, R., Lee, S. C., Su, Q., Melvin, D., ... Liu, P. (2007). A recombineering based approach for high-throughput conditional knockout targeting vector construction. *Nucleic Acids Research*, 35(8), e64. doi:10.1093/nar/gkm163
- Copeland, N. G., Jenkins, N. a, & Court, D. L. (2001). Recombineering: a powerful new tool for mouse functional genomics. *Nature Reviews. Genetics*, 2(10), 769–79. doi:10.1038/35093556
- Costantino, N., & Court, D. L. (2003). Enhanced levels of lambda Red-mediated recombinants in mismatch repair mutants. *Proceedings of the National Academy of Sciences of the United States of America*, 100(26), 15748–53. doi:10.1073/pnas.2434959100
- Court, D. L., Sawitzke, J. a, & Thomason, L. C. (2002). Genetic engineering using homologous recombination. *Annual Review of Genetics*, 36, 361–88. doi:10.1146/annurev.genet.36.061102.093104
- Datsenko, K. a, & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proceedings of the National Academy of Sciences of the United States of America*, 97(12), 6640–5. doi:10.1073/pnas.120163297
- Datta, S., Costantino, N., & Court, D. L. (2006). A set of recombineering plasmids for gram-negative bacteria. *Gene*, 379, 109–15. doi:10.1016/j.gene.2006.04.018
- Ellis, H. M., Yu, D., DiTizio, T., & Court, D. L. (2001). High efficiency mutagenesis, repair, and engineering of chromosomal DNA using single-stranded oligonucleotides. *Proceedings of the National Academy of Sciences of the United States of America*, 98(12), 6742–6. doi:10.1073/pnas.121164898
- Gates, C. a, & Cox, M. M. (1988). FLP recombinase is an enzyme. *Proceedings of the National Academy of Sciences of the United States of America*, 85(13), 4628–32. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/24456598>
- Hillyar, C. R. T. (2012). Review Genetic recombination in bacteriophage lambda, 5, 1–7.

- Hornsey, M., Ellington, M. J., Doumith, M., Scott, G., Livermore, D. M., & Woodford, N. (2010). Emergence of AcrAB-mediated tigecycline resistance in a clinical isolate of *Enterobacter cloacae* during ciprofloxacin treatment. *International Journal of Antimicrobial Agents*, 35(5), 478–481. doi:10.1016/j.ijantimicag.2010.01.011
- Jung, M. Y., Ng, C. Y., Song, H., Lee, J., & Oh, M. K. (2012). Deletion of lactate dehydrogenase in *Enterobacter aerogenes* to enhance 2,3-butanediol production. *Applied Microbiology and Biotechnology*, 95, 461–469. doi:10.1007/s00253-012-3883-9
- Karstentischer, B., von Einem, J., Kaufer, B., & Osterrieder, N. (2006). Two-step Red-mediated recombination for versatile high-efficiency markerless DNA manipulation in *Escherichia coli*. *BioTechniques*, 40(2), 191–197. doi:10.2144/000112096
- Keefer, L. M., Piron, M. a, & De Meyts, P. (1981). Human insulin prepared by recombinant DNA techniques and native human insulin interact identically with insulin receptors. *Proceedings of the National Academy of Sciences of the United States of America*, 78(3), 1391–1395. doi:10.1073/pnas.78.3.1391
- Lee, E. C., Yu, D., Martinez de Velasco, J., Tessarollo, L., Swing, D. a, Court, D. L., ... Copeland, N. G. (2001). A highly efficient *Escherichia coli*-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics*, 73(1), 56–65. doi:10.1006/geno.2000.6451
- Lin, S., Ewen-Campen, B., Ni, X., Housden, B. E., & Perrimon, N. (2015). In Vivo Transcriptional Activation Using CRISPR/Cas9 in *Drosophila*. *Genetics*, 201(2), 433–442. doi:10.1534/genetics.115.181065
- Liu, P., Jenkins, N. a, & Copeland, N. G. (2003). A highly efficient recombineering-based method for generating conditional knockout mutations. *Genome Research*, 13(3), 476–84. doi:10.1101/gr.749203
- Mansour, S. L., Thomas, K. R., Deng, C. X., & Capecchi, M. R. (1990). Introduction of a lacZ reporter gene into the mouse int-2 locus by homologous recombination. *Proceedings of the National Academy of Sciences of the United States of America*, 87(19), 7688–92. Retrieved from <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=54813&tool=pmcentrez&rendertype=abstract>
- Mosberg, J. a., Lajoie, M. J., & Church, G. M. (2010). Lambda Red Recombineering in *Escherichia coli* Occurs Through a Fully Single-Stranded Intermediate. *Genetics*, 186(3), 791–799. doi:10.1534/genetics.110.120782
- Murphy, K. C. (1998). Use of Bacteriophage λ Recombination Functions To Promote Gene Replacement in *Escherichia coli* Use of Bacteriophage Recombination Functions To Promote Gene Replacement in *Escherichia coli*, 180(8).
- Narayanan, K., & Chen, Q. (2011). Bacterial artificial chromosome mutagenesis using recombineering. *Journal of Biomedicine & Biotechnology*, 2011, 971296. doi:10.1155/2011/971296

- Oppenheim, A. B., Rattray, A. J., Bubunencko, M., Thomason, L. C., & Court, D. L. (2004). In vivo recombineering of bacteriophage lambda by PCR fragments and single-strand oligonucleotides. *Virology*, 319(2), 185–9. doi:10.1016/j.virol.2003.11.007
- Rivero-Müller, A., Lajić, S., & Huhtaniemi, I. (2007). Assisted large fragment insertion by Red/ET-recombination (ALFIRE) - An alternative and enhanced method for large fragment recombineering. *Nucleic Acids Research*, 35(10). doi:10.1093/nar/gkm250
- Sawitzke, J. a, Thomason, L. C., Costantino, N., Bubunencko, M., Datta, S., & Court, D. L. (2007). Recombineering: in vivo genetic engineering in *E. coli*, *S. enterica*, and beyond. *Methods in Enzymology*, 421(06), 171–99. doi:10.1016/S0076-6879(06)21015-2
- Seier, T., Padgett, D. R., Zilberberg, G., Sutera, V. a, Toha, N., & Lovett, S. T. (2011). Insights into mutagenesis using *Escherichia coli* chromosomal lacZ strains that enable detection of a wide spectrum of mutational events. *Genetics*, 188(2), 247–62. doi:10.1534/genetics.111.127746
- Sergueev, K., Yu, D., Austin, S., & Court, D. (2001). Cell toxicity caused by products of the p L operon of bacteriophage lambda, 272, 227–235.
- Sharan, S. K., Thomason, L. C., Kuznetsov, S. G., & Court, D. L. (2009). Recombineering: a homologous recombination-based method of genetic engineering. *Nature Protocols*, 4(2), 206–23. doi:10.1038/nprot.2008.227
- Swingle, B., Markel, E., Costantino, N., Bubunencko, M. G., Cartinhour, S., & Court, D. L. (2010). Oligonucleotide recombination in Gram-negative bacteria. *Molecular Microbiology*, 75(1), 138–48. doi:10.1111/j.1365-2958.2009.06976.x
- Thomason, L. C., Oppenheim, A. B., & Court, D. L. (2009). Bacteriophages, 501, 239–251. doi:10.1007/978-1-60327-164-6
- Thomason, L., Court, D. L., Bubunencko, M., Costantino, N., Wilson, H., Datta, S., & Oppenheim, A. (2007). Recombineering: genetic engineering in bacteria using homologous recombination. *Current Protocols in Molecular Biology / Edited by Frederick M. Ausubel ... [et Al.]*, Chapter 1, Unit 1.16.
- Tilghman, S. M., Tiemeier, D. C., Polsky, F., Edgell, M. H., Seidman, J. G., Leder, a, ... Leder, P. (1977). Cloning specific segments of the mammalian genome: bacteriophage lambda containing mouse globin and surrounding gene sequences. *Proceedings of the National Academy of Sciences of the United States of America*, 74(10), 4406–4410. doi:10.1073/pnas.74.10.4406
- Yu, D., Ellis, H. M., Lee, E., Jenkins, N. A., Copeland, N. G., & Court, D. L. (2000). An efficient recombination system for chromosome engineering in *Escherichia coli*, 97(11).