

# Characterizing a Lambda Red Recombinase Induced Presumptive Partial Deletion of *lacI* in *Escherichia coli* C29 that Affects Regulation of $\beta$ -galactosidase production

ADA WOO

*Department of Microbiology and Immunology, UBC*

The  $\lambda$  Red recombination system was used in this study in an attempt to inactivate the *lacI* gene in *Escherichia coli* C29 cells. The proposed model retained the first 41 amino acids of the *lacI* gene, and replaced the rest of the gene with a linear double-stranded DNA PCR product that confers kanamycin resistance. The PCR products encode a kanamycin cassette with *lacI* flanking region. The initial step was to transform wild type *E.coli* C29 with plasmid pKD46 using electroporation. This transformation was highly successful and yielded ampicillin resistance cells conferring pKD46. pKD46 encodes enzymes required for the recombination process. The second step was to introduce the PCR products into cells conferring plasmid pKD46 again using electroporation. Since the PCR products contain *lacI* flanking regions, recombination should occur at the homologous *lacI* sequence in the bacterial chromosome. The second transformation produced Kan<sup>R</sup> *E.coli* C29.  $\beta$ -galactosidase activity of Kan<sup>R</sup> *E.coli* C29 cells was measured using the  $\beta$ -galactosidase assay. No significant difference in  $\beta$ -galactosidase activity was observed between Kan<sup>R</sup> and wild type *E.coli* C29 cells. Evidence showed that *lacI* was still active and the partial deletion (if it occurred) of the *lacI* gene was not enough to inactivate the protein. Further studies are needed to determine if the  $\lambda$  red recombination system was successful in inserting the kanamycin gene in place of the distal part of *lacI*.

The *lac* operon is a well studied example of a series of repressor regulated genes. The *lac* operon encodes for LacI, a promoter, an operator, LacZ, LacY and LacA in sequential order. The *lacZ* is the gene responsible for the production of  $\beta$ -galactosidase.  $\beta$ -galactosidase cleaves lactose into galactose and glucose so that the sugars can be further broken down into an energy source used by the organism. The expression of the operon can be regulated by the LacI repressor protein via a global regulatory mechanism (2). Since LacZ is encoded within the *lac* operon, LacI also regulates *lacZ* and consequently the production of  $\beta$ -galactosidase. In the presence of glucose, *lacI* is transcribed. The active LacI protein binds to the operator of the *lac* operon to inhibit transcription of preceding genes. When glucose level is low, LacI is inactivated by binding to an inducer. Inactivated LacI is unable to bind to the operator, allowing the preceding genes to be transcribed (8). The LacI repressor protein is composed of four identical subunits. Each subunit is 360 amino acids in size. The first 59 amino acids at the amino-terminal end acts as the DNA and operator binding sites. The next 60-360 amino acids contain the binding site for inducers and dimerization sites (7). Dimerization is thought to be a major component in the inhibition of transcription of the preceding genes; therefore deletion of the last 300 amino acids should be significant enough to inactivate LacI. Furthermore,

deletion of the last 300 amino acids would also remove binding site for inducers such as allolactose and IPTG. The resulting LacI mutant should produce the same amount of *lac* gene products in the presence and absence of an inducer.

A method that is used for performing gene replacements in *E.coli* is the phage  $\lambda$  Red pathway. The  $\lambda$  Red pathway allows linear double-stranded DNAs as short as 30 bases to replace the targeted sequence in the chromosome (1). The  $\lambda$  Red system requires two main components: 1) a defective prophage in which most of the  $\lambda$  genes has been deleted except the recombination genes  $\gamma$ - $\beta$ -exo (pKD46)(1). 2) a region of the plasmid amplified with PCR and the double stranded fragment carrying an antibiotic resistance cassette flanked by sequences homologous to the sequences to be replaced in the targeted sequence in the chromosome (10). The three proteins ( $\gamma$ - $\beta$ -exo) encoded by the defective prophage are the key enzymes for the replacement process. The  $\gamma$  protein inhibits the RecBCD enzyme allowing the use of linear DNA fragment. The  $\beta$  protein ( $\beta$ -gene product) binds to single stranded DNA exposed by Exo and promotes synapse formation and strand exchange with another DNA. Exo is an exonuclease which degrades one strand of a double-stranded DNA, and exposes a single strand for strand invasion (10).

When Jager *et al.* used the  $\lambda$ -Red system to knockout the last 957 nucleic bases of the *lacI* gene, no amber mutation was introduced between the *lacI* sequence and the kanamycin cassette. Jager *et al.* did not see a difference in LacI inhibition between the wild type and the mutant *E. coli*. The absence of a stop codon suggests that a LacI peptide more than 41 amino acids long was made and the additional amino acids might have been associated with the inhibition of the *lacZ* gene. This study intended to find out the ability of *lacZ* regulation by only the first 41 amino acids of the *lacI* gene. The first 41 amino acids of the LacI were retained by introducing an amber mutation in the correct reading frame to replace the 123<sup>th</sup> nucleic bases when the kanamycin cassette was inserted. This allows transcription to terminate at the end of the 124<sup>th</sup> nucleic base and only a 41 amino acid peptide will be translated.

### MATERIALS AND METHODS

**Plasmids.** Plasmid pKD46 (Fig. 1) is 6329bp in size. It expresses the  $\lambda$  Red system under control of a well-regulated promoter to avoid unwanted recombinational events under non-inducing conditions (3). Plasmid pKD46 encodes the 3 essential genes ( $\gamma, \beta, \text{exo}$ ) for the recombinant system as well as an ampicillin resistant gene (*bla*). The 3941 bp plasmid pACYC177 (Fig. 2) encodes a kan gene, coding for the aminoglycoside 3'-phosphotransferase that confers resistance to kanamycin (source - transposon Tn903) (5). Plasmid pACYC177 was used a template in the PCR reaction for amplification of the kanamycin cassette.

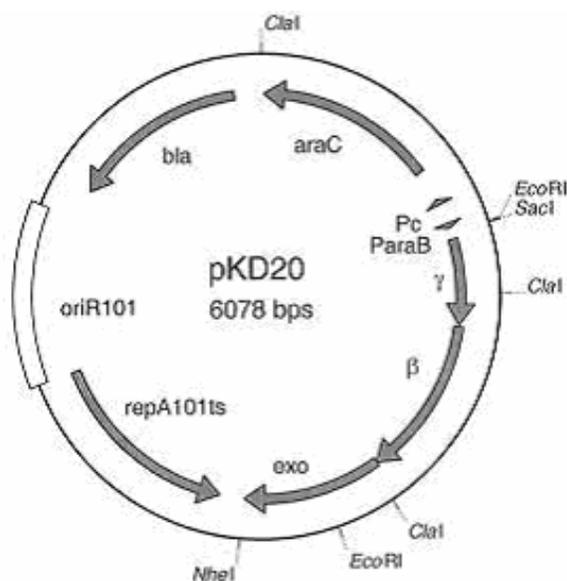


FIG. 1 Red recombinase expression plasmid pKD20 (shown in the figure) is 251bp smaller than pKD46 (not shown). Plasmid pKD46 is identical to pKD20, with the addition of  $\lambda$  tL3 terminator (5).

**Media.** Broth cultures of *E. coli* C29 cells were grown at 37°C with shaking Luria Bertain (LB) Broth; LB broth is comprised of 1%w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl.

**Cells.** *Escherichia coli* K12, strain C29 cells (obtained from MICB 447 laboratory) were transformed with pKD46 plasmid isolate by Jaeger *et al.* (6) (obtained from MICB 447 laboratory, UBC) Amp<sup>R</sup> cells (transformed *E. coli* C29 cells harboring the plasmid pKD46) were selected on solid LB plates containing a final working concentration of 100  $\mu$ g/mL ampicillin. Kan<sup>R</sup> cells (transformed Amp<sup>R</sup> *E. coli* C29 cells harboring the PCR-kanamycin cassette product) were selected on solid LB plates containing a final working concentration of 50  $\mu$ g/mL Kanamycin. Kan<sup>R</sup> cells were then plated on solid inducible LB plates and solid non-inducible LB plates to test for the production of  $\beta$ -galactosidase. Solid inducible LB plates contain 50  $\mu$ g/mL Kanamycin, 1.6 mg/plate of IPTG, and 1.6 mg/plate of X-gal. Solid non-inducible LB plates contained 50 $\mu$ g/mL Kanamycin, and 1.6 mg/plate of X-gal. IPTG and X-gal were spread plated onto solid LB Kanamycin plates.

**Media used in  $\beta$ -galactosidase assay.** Broth cultures of Kan<sup>R</sup> cells (transformed Amp<sup>R</sup> *E. coli* C29 cells transformed with the PCR-kanamycin cassette product) were grown in modified Luria Broth at 37°C with shaking; modified LB broth is comprised of 1%w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl, 0.2% v/v glycerol and 50  $\mu$ g/mL Kanamycin. Kan<sup>R</sup> cells were also grown in induction media containing modified LB broth with 1mM IPTG. Tris buffer used for the assay was at 20mM and adjusted to pH 8.

**Primers and PCR.** The sequence of forward primer was 5' TCTGCGAAAACGCGGGA AAAAGTGGAAAGCGGTAATGCG TTGTGCGGAAGATGCG-3'. Sequence of the reverse primer was 5'TACTGCCCCGCTTCCAGTCGGGAAACCTGTCGTGCGTGA TCTGATCCTTCAACTC-3'. The forward primer was modified from the forward primer used by Jaeger *et al.* (6), where changes were made at the 34<sup>th</sup>-36<sup>th</sup> nucleotide. The forward primer was designed to contain last 33 nucleotides of *E. coli* C29 *lacI* DNA binding domain gene, followed by the stop codon "TAA" and 20 nucleotides flanking the kanamycin resistance cassette contained in the pACYC177 plasmid. The reverse primer was designed to contain 36 nucleotides of *E. coli* C29 *lacI* gene, followed by 20 nucleotides of *E. coli* C29 flanking the kanamycin resistance cassette contained in the pACYC177 plasmid. The reverse primer was constructed by Jaeger *et al.* (6) (obtained from MICB 447 laboratory, UBC). The primers were BLAST against the *E. coli* K12 total genome, pACYC177 plasmid and pKD46 plasmid to determine specificity. The forward primer was synthesized by the Nucleic Acid Protein Synthesis Unit (NAPS, UBC). The dried pellet received from NAPS was resuspended in sterile distilled water. The concentration of the primer was determined by OD<sub>260</sub> value.

The optimal condition for the PCR reaction of the kanamycin resistance cassette was obtained by running samples of different concentration of primers and pACYC177 template DNA while all temperature and incubation times remained the same. The optimal condition for amplification of the kanamycin resistance cassette-*lacI* construct was 0.25  $\mu$ l Taq Polymerase (Invitrogen Life Technologies), 0.025  $\mu$ g of pACYC177 template DNA, and 0.2  $\mu$ l of each primer (15  $\mu$ M). Plasmid pACYC177 was isolated by Jaeger *et al.* (6) (obtained from MICB 447 laboratory, UBC). PCR conditions were as follows: an initial denaturation of 95°C for 4 minutes, 40 cycles of 95°C for 45 seconds, 50°C for 45 seconds and 72°C for 1 minute, 30 seconds and final extension of 72°C for 10 minutes.

PCR products were electrophoresed on 1% agarose gel at 100V for 60 minutes to confirm the presence and size of the PCR product. The PCR products were then purified using the MinElute PCR Purification Kit (Qiagen Inc.).

### Preparation of Electrocompetent Cells and Transformations.

**Electrocompetent *E. coli*** - *E. coli* C29 cells were grown in 500 mL culture overnight in LB broth at 37°C with aeration. When the turbidity of the culture reached 1.3 OD<sub>660</sub>, cells were chilled on ice for 20 minutes. After incubation, cells were centrifuged at 4000 x g for 15 minutes. The supernatant was discarded, and the pellet was

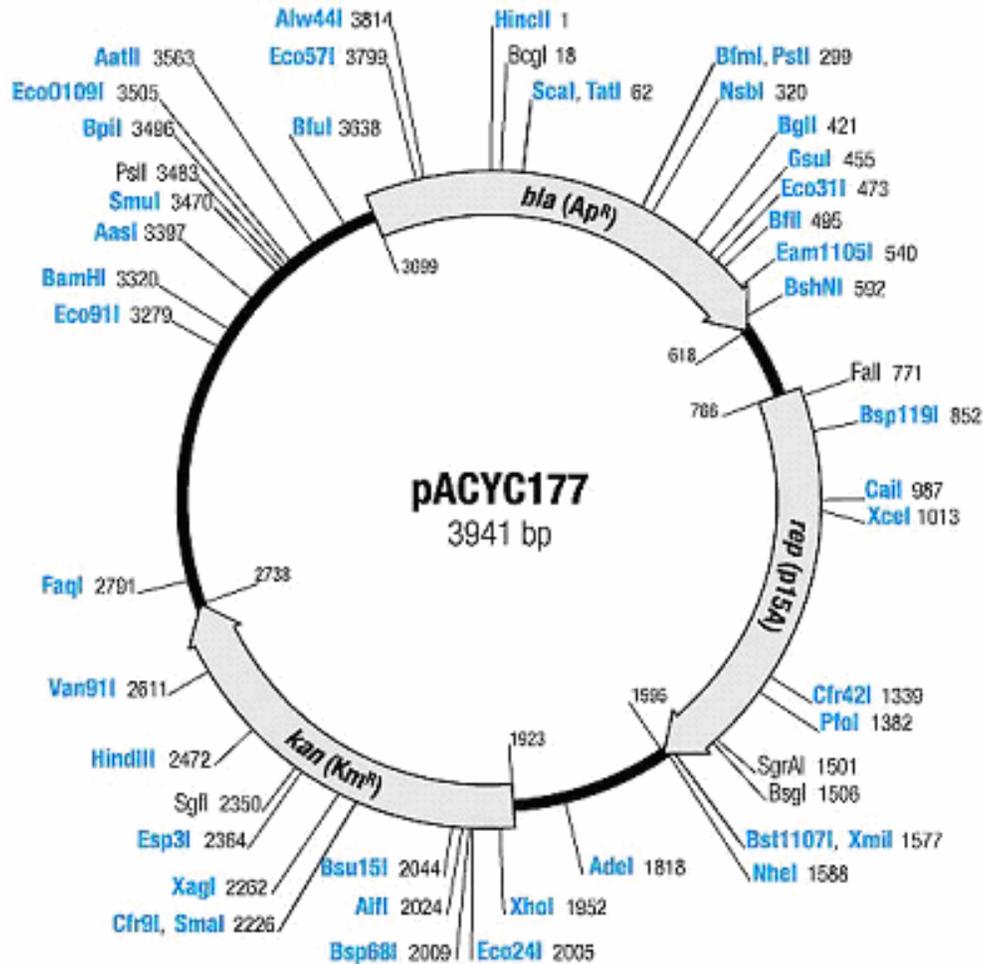


FIG. 2 Restriction digest map of plasmid pACYC177. Plasmid pACYC177 confers the kanamycin resistance and was used as DNA template for PCR reactions (6).

resuspended in 500 mL of ice cold 10% glycerol. Resuspended cells were centrifuged again at 4000 x g for 15 minutes. Again supernatant was discarded and pellet was resuspended in 250 mL of ice cold 10% glycerol. Resuspended cells were centrifuged again at 4000 x g for 15 minutes, supernatant was discarded and pellet was resuspended in 20 mL of ice cold 10% glycerol. Cells were centrifuged for the fourth time at 4000 x g for 15 minutes, supernatant was removed and pellet was resuspended in 2 mL of ice cold 10% glycerol. After the final resuspension, cells were stored in 100 µl aliquots at -80°C. Note that all centrifugation were performed at 4°C.

The same procedure was used for Amp<sup>R</sup> *E.coli* C29 except the cells were grown in 100 mL cultures and to the OD<sub>660</sub> of 1.0. The amounts of all other reagents added were adjusted accordingly (1/5 of the volume described above for transforming electrocompetent *E.coli* C29).

**Transformation** – 100 µL of electrocompetent *E.coli* C29 cells were thawed on ice. 40 µL of the thawed electrocompetent *E.coli* C29 cells were mixed with 2 µL of plasmid pKD46 and incubated on ice for 1 minute. The mixture was then transferred to a pre-chilled BIO-RAD 0.2cm cuvette. Using the BIO-RAD MicroPulser, the mixture was pulsed at 2.5 kV for 1 second (program EC2), as per manufacturer's protocol. Electroporated cells were resuspended in 1 mL of LB broth (pre-heated to 37°C) and transferred to culture tube. Cells were grown at 37°C with aeration for 1 hour. After incubation

100 µL of culture was used for spread plating onto a solid LB ampicillin plate.

The same conditions were used for the second transformation except Electrocompetent Amp<sup>R</sup> *E.coli* C29 cells, and PCR DNA products were used in place of electrocompetent *E.coli* C29 cells and plasmid pKD46. Transformed cells were selected on solid LB kanamycin plate.

**β – galactosidase assay** – 10 of the 29 kanamycin resistant colonies were randomly chosen for further analysis of β – galactosidase activity. Each colony was grown in 1 - 5mL culture of modified LB broth and 1 – 5mL culture of induction LB media. Wild type *E.coli* C29 were grown overnight in LB broth plus 10% glycerol and LB broth plus 10% glycerol and 1 mM isopropylthiogalactoside (IPTG). Cultures were incubated overnight at 37°C with aeration. After incubation, 2 mL of each culture was removed from culture tube and the OD<sub>660</sub> was measured. 200 µL of toluene was added to the remaining 3 mL of overnight cultures, followed by vortexing. The samples were incubated at room temperature until the toluene and the cell culture separated into two phases. 100 µL of each culture was transferred into clean labeled reaction tubes, along with 300 µL of sterile distilled water and 1.2 mL of tris buffer. The reaction time commenced as soon as 200 µL of 5 mM orthonitrophenogalactoside (ONPG) was added to the reaction tube. The enzyme reaction was carried out at 37°C and stopped with 2 mL of 0.6M sodium carbonate.

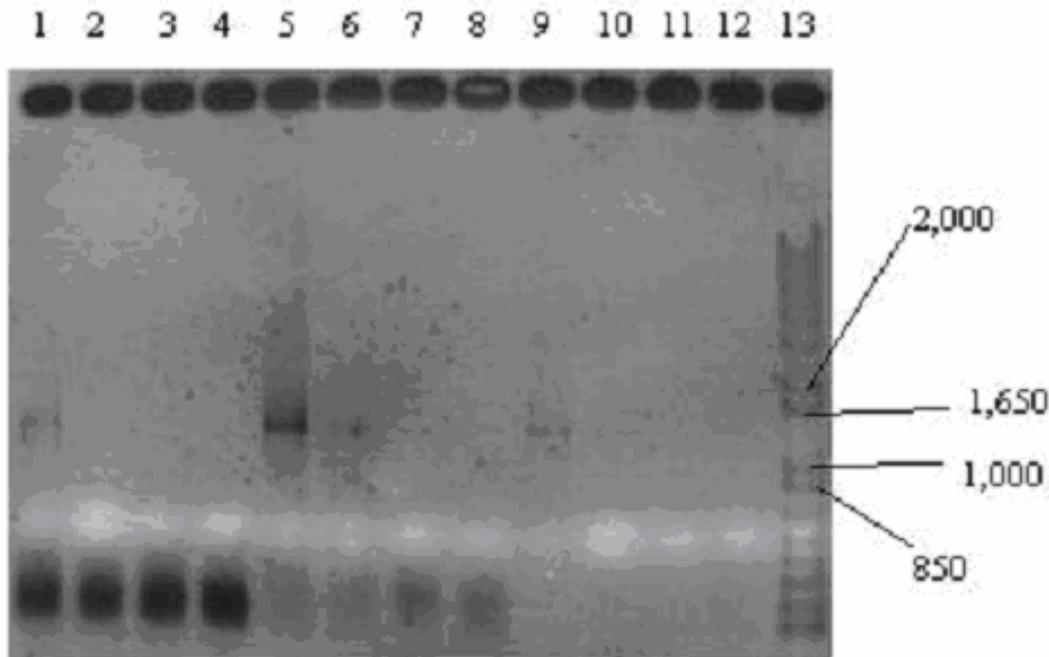


FIG. 3 Gel Electrophoresis of PCR DNA products. Lane 1-4, 7, 8, 10, 11, 12 are PCR conditions that yield very low or no PCR products. Lane 5 (0.25 $\mu$ L Taq Polymerase (Invitrogen Life Technologies), 0.025 $\mu$ g of pACYC177 template DNA, and 0.2 $\mu$ L of each primer (15 $\mu$ M).) – condition that yield largest amounts of PCR products. Lane 6 (0.25 $\mu$ L Taq Polymerase (Invitrogen Life Technologies), 2.5ng of pACYC177 template DNA, and 0.2 $\mu$ L of each primer (15 $\mu$ M).) – condition that yield low amounts of PCR products. Lane 9 (0.25 $\mu$ L Taq Polymerase (Invitrogen Life Technologies), 0.025 $\mu$ g of pACYC177 template DNA, and 0.02 $\mu$ L of each primer (15 $\mu$ M).) – conditiona the yield low amounts of PCR products. All PCR reaction was done at 95°C for 180 seconds, 95°C for 45 seconds, 50°C for 45 seconds, 72°C at 90 seconds, and 72°C for 10 minutes; steps 2-4 were repeated for 40 cycles.

Enzyme reaction was detected by measuring the absorbance of the nitrophenol product at 420nm. Results from the  $\beta$ -galactosidase assay were converted into specific activity using formulas outlined by Ramey (9). The equation for the activity (9) uses the molar extinction coefficient for ONPG in the Spectronic 20+ cuvettes, as oppose to Ultraspec 3000 cuvettes which were used in this experiment; therefore the real specific activity should be higher than calculated values because the path length of the Ultraspec 3000 cuvettes were shorter than that of Spectronic 20+ cuvettes. Since only relative values were needed to compare activity among the mutants, and all values were obtained using the same formula, further manipulation was not needed.

IPTG is an inducer of the *lac* operon, that promotes the production of  $\beta$ -galactosidase (2). IPTG is a common substance used to increase  $\beta$ -galactosidase in absence of lactose. In practice, it is beneficial to have an *E.coli* strain that constantly expresses  $\beta$ -galactosidase, because the strain would eliminate the used of inducing agent in growth media. The rate of  $\beta$ -galactosidase production was measured by assaying the specific activity using o-nitrophenol  $\beta$ -galactoside (ONPG) as a substrate. ONPG is cleaved by  $\beta$ -galactosidase to yield a coloured product and the concentration can be determined using a spectrophotometer (2). Using the cell concentrations of the cultures and  $\beta$ -galactosidase activity measured by the assay, specific activity was then determined. Specific activity was used to compare enzyme activity because the values account for the differences in cell concentrations.

## RESULTS

The forward primer was BLAST against *E.coli* K12 (U00096). The Expect Value where the primer is

homologous to the desired *lacI* region in the organism was  $3 \times 10^{-12}$ . The next smallest Expect Value was 0.61. When the forward primer was BLAST against plasmid pACYC177 (X06402), two hits were found with the Expect Value of  $2 \times 10^{-7}$ . The two hits were located at 5' and 3' ends of the kanamycin cassette. Homology of the forward primer and plasmid pKD46 (AY048746) was very low as suggested by the smallest Expect Value of 0.9 when BLAST search was performed.

When the reverse primer was BLAST against *E.coli* K12 (U00096) the lowest Expect Value was found to be  $5 \times 10^{-14}$  and is homologous to the 3' end of the *lacI* gene. The next smallest Expect Value found was 0.039. BLAST search reported two Expect Values of  $2.2 \times 10^{-9}$  when the reverse primer was matched with plasmid pACYC177 (X06402). Again the locations of the matches were located at 5' and 3' ends of the kanamycin cassette. An Expect Value of 0.9 was found when the reverse primer was BLAST against plasmid pKD46 (AY048746).

*E. coli* C29 that were transformed with pKD46, and colonies were recovered when grown on solid LB-ampicillin plates, indicating that the first transformation was successful.

PCR was performed using designed primers and plasmid pACYC177 to amplify the kanamycin resistant

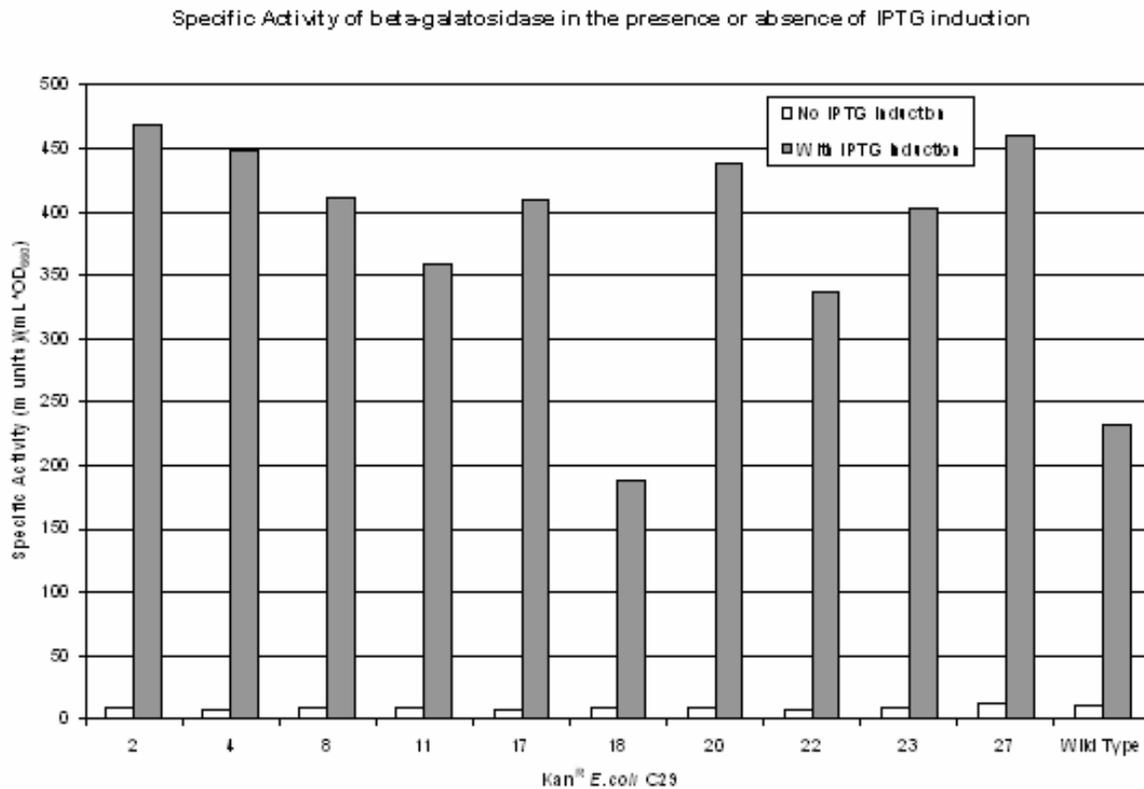


FIG. 4 Specific Activity of  $\beta$ -galactosidase in the presence or absence of IPTG induction. Cultures were grown in the presence and absence of IPTG overnight, and  $\beta$ -galactosidase activity was measured using the  $\beta$ -galactosidase assay as described above. The wild type culture is *E. coli* C29 initially obtained from MICB447 laboratory prior to any transformation.

cassette. Lane 5 (Fig. 3) shows the PCR product to be approximately 1200bp when compared with the 1kb plus ladder on lane 13. The expected size of the PCR product is 1169bp. *Amp<sup>R</sup> E. coli* C29 cells were transformed with PCR DNA product. A total of 29 colonies grew on selective solid LB-kanamycin plates. PCR conditions have successfully cloned the kanamycin resistant cassette and transformation of the PCR DNA product was successful. The 29 isolated colonies were plated on IPTG-X-gal-Kan-LB plates and X-gal-Kan-LB plates. All 29 clones appeared blue on both sets of plates. 10 colonies were randomly selected for the  $\beta$ -galactosidase assay.

The purpose of the first transformation was to introduce plasmid pKD46 into wild type *E. coli* C29. pKD46 (Fig. 1) confers the  $\gamma$ -bet-exo proteins which are essential for activation of the  $\lambda$  Red recombination system. Plasmid pKD46 also confers ampicillin resistance; therefore resulting transformants will be *Amp<sup>R</sup> E. coli* C29. First transformation was successful. Successfully transformed cells were able to grow on LB plates containing 100 $\mu$ g/mL of ampicillin. A single colony from the LB plates was randomly selected and

then grown in batch culture and finally prepared for second transformation.

The second transformation was to introduce the PCR products into *Amp<sup>R</sup> E. coli* C29 cells. The PCR products contained region of the pACYC177 plasmid (Fig. 2) carrying the kanamycin resistance cassette flanked by sequences homologous to the sequences to be replaced in the bacterial chromosome (10). With the presence of the  $\gamma$ -bet-exo proteins encoded by plasmid pKD46, the linear double-stranded DNA PCR products can attach to homologous regions of the *lacI* gene, replacing the *lacI* gene with an active kanamycin cassette. Successful recombinant mutants are *Kan<sup>R</sup> LacI<sup>-</sup> E. coli* C29. Cells that were transformed with PCR products were selected by plating cells on LB plates containing 30 $\mu$ g/mL kanamycin. Selective kanamycin plates can only select for *Kan<sup>R</sup>* mutants but not *Kan<sup>R</sup> LacI<sup>-</sup>* cells. *Kan<sup>R</sup> E. coli* C29 cells were also plated on LB plates containing X-gal (5-Bromo-4-Chloro-3-Indolyl- $\beta$ -D-Galactopyranoside) for a quick colorimetric detection of  $\beta$ -galactosidase enzyme activity. In the presence of  $\beta$ -galactosidase activity, X-gal is cleaved and colonies become blue. The X-gal test can only determine the presence or absence of  $\beta$ -

galactosidase activity and is unable to determine the concentration of the amount enzyme activity. Since X-gal was spread onto the LB agar plates, the concentration of X-gal was not likely to be evenly distributed through out the plate. Regions of higher concentration of X-gal resulted in colonies that develop a darker blue colour.

Figure 4 shows the specific activity in each condition of each clone. In all cases it was clear that the presence of galactosidase activity was dependent on the inducer as if the *LacI* was still wild type in activity. This observation was further confirmed using the Wilcoxon signed-rank test at 95% confidence level.

## DISCUSSION

The most important variable component in the  $\lambda$  red recombination system is the PCR products. The PCR products define the specificity of the site of recombination by sequence homology at the ends of the DNA fragments. The homology sequence determines the location of the target chromosome where the PCR products will be inserted. Hence if the flanking regions are homologous to several regions of the chromosome, recombination can occur at all homologous regions. Therefore specificity of the homologous flanking sequence is extremely important.

Homology and specificity was determined by performing BLAST searches against *E.coli* K12, plasmid pACYC177 and pKD46. Location of the homologous sequence and the Expect values were used to determine specificity of the primers. The Expect value describes the random background noise that exists for matches between the sequences. The lower the Expect Value is the more significant the match. Essentially, if the Expect Value is zero, then there is no chance of the match to occur by chance. The most important Expect Values to look at, were the 2 primers versus *E.coli* K12. The Expect Values of primers versus *E.coli* K12 could help determine the specificity of the recombination. Since the Expect Values of the primers versus *E.coli* K12 were low at the *lacI* region, then the PCR products were specific and recombination would occur at the specified regions. It is also important for the primers have low homology sequence with plasmid pKD46. If primers match sequences in pKD46, then recombination into the plasmid could occur resulting in Kan<sup>R</sup> *E.coli* C29 without *LacI* disruption. Specificity of primers versus plasmid pACYC177 determines the purity of the PCR products. If primers are not specific for the kanamycin cassette, then more than one PCR product would be made. Both primers were detected to have a high specificity at both 5' and 3' ends of the kanamycin cassette. Because the kanamycin cassette came from a transposon, repeated

sequences were found on either ends of the cassette. In turn, the primers were able to bind to either ends of the cassette. In theory, if the primers were reversely bound to the plasmid, the reaction would not be able to continue because base pairing only occurs in one direction.

Taq DNA polymerase used in the PCR reaction lacks proofreading abilities. Potentially, the enzyme makes mismatch to the DNA template and the resulting PCR production might not be identical to the original DNA template. In a PCR reaction, productions are reused as templates; therefore a build-up of mispairing can occur. Error of Taq polymerase depends of the assay used. From literature, error rate of Taq polymerase from Invitrogen Life Technologies has an error rate range of  $1.1 \times 10^{-4}$  to  $8.9 \times 10^{-5}$  errors/bp (product information, Invitrogen Life Technologies website). If errors occurred within the flanking regions, then homology of the flanking region and the targeted *lacI* region would be decreased. Decrease in homology will decrease the number of successful recombinations. Considering the size of the homology sequence and the error rate, the errors would probably not be significant enough to cause a great decrease of successful recombination events. Errors caused by the Taq DNA polymerase can be introduced any in the PCR product. If an error occurred in the introduced stop codon or an addition/deletion mutation was introduced to cause a change in the reading frame of the introduced stop codon, then *LacI* mutants with more than 41 amino acids might be made. In such a case, the addition amino acids might affect the binding ability of the mutated *LacI*.

The designed kanamycin cassette with *lacI* flanking sequence is 1169bp in length. Size of the PCR product was determined using gel electrophoresis to ensure the products encode the designed regions. Sizes of the PCR products were estimated by comparing with the 1kb plus ladder (Fig. 3). The estimated size of the PCR was 1200pb which is roughly the expected size. Size determination is not the best method to identify the desire PCR product. To better endure the correct product is produced, a digest should be done followed by gel electrophoresis. The PCR product can be digested using one or more restriction enzyme that will cut within the cassette. Using a restriction map (Fig. 2) the size of the cut fragments could be determined. If the size of the cut fragments matches the expected values, then there is strong evidence that the PCR products are the designed kanamycin cassette. Although a digest mapping analysis was not performed, size determination and successful Kan<sup>R</sup> *E.coli* C29 are good indication that the PCR products contained the designed kanamycin cassette.

Kan<sup>R</sup> *E.coli* C29 mutants that lack a functional *lacI* gene were detected using the  $\beta$ -galactosidase assay.

The activity of the  $\beta$ -galactosidase enzyme can also be determined using this assay. Activity of  $\beta$ -galactosidase enzyme produced by the wild type *E.coli* C29 was compared with Kan<sup>R</sup> *E.coli* C29. A successful transformant will produce the same amount of  $\beta$ -galactosidase in the presence and absence of IPTG. Unfortunately, the results from the  $\beta$ -galactosidase assay did not give evidence to support that a Kan<sup>R</sup>Lac<sup>-</sup>*E.coli* C29 mutant was created. Most mutants produced more  $\beta$ -galactosidase than the wild type *E.coli* C29 when induced by IPTG (Fig. 4). To confirm that the induced samples really produced more  $\beta$ -galactosidase than the uninduced samples, a statistical analysis call a Wilcoxon signed-rank test can be performed (4). The rank test further confirms that there is a difference between the paired data. Each mutant occurred from a different recombinant event; therefore should be treated as a separate strain. Although the  $\beta$ -galactosidase activity appears to be higher in the mutants compared with the wild type *E.coli*, variance in the data cannot be determined when the assay was only performed once. To determine weather there was a significant difference between induced  $\beta$ -galactosidase activity of mutants and wild type *E.coli* a statistical test could be performed. To perform any statistical analysis, the assay must be performed more than once. Statistical analysis cannot be performed using 1 set of data. At this point, there is no evidence to support that there is a significant difference in  $\beta$ -galactosidase activity between the mutants and wild type *E.coli* C29.

The purpose of phenotype of the mutant was Kan<sup>R</sup> Lac<sup>-</sup> *E.coli* C29. This was not observed. Evidence showed that the resulting mutant has only the Kan<sup>R</sup> phenotype. There was no evidence supporting a successful recombination in which *lacI* gene has become dysfunctional. Resulting kan<sup>R</sup> *E.coli* C29 suggested that the kanamycin cassette was inserted elsewhere in the genome without disrupting the *lacI* function. Since in theory, successful recombination will retain the first 41 amino acids of LacI, if successful recombination did occur, then the first 41 amino acids plays a very important role in the regulation of the operon. It would suggest that using the first 41 amino acids would be enough to produce a functional LacI protein that can regulate the operon if the kanamycin is inserted properly.

The unsuccessful disruption of the *lacI* gene can also be explained by a rare event that could happen to linear DNA. The ends of the linear PCR product can react with each other and become circularized. Circularized PCR product can only interact with one site in the bacterial chromosome. Much like a transposon, the circularized PCR product can integrate into the one recognition site. If the circularized PCR product was inserted into the 3' end of the gene, then

the surviving mutant will become kanamycin resistant but retain a non-disrupted, active *lacI* gene. After reviewing the results obtained from the BLAST searches, circularization is highly possible. Homologous regions to the kanamycin flanking regions of both primers were found on both ends of the kanamycin cassette. The resulting PCR products from these primers have ends that are homologous; therefore are very likely that the ends will bind to each other to form a circularized piece of DNA. The kanamycin cassette originated from a transposon Tn903 (7). As a property of a transposon, inverted repeats are located at both ends of the kanamycin cassette. These inverted repeats will increase the chance of circularization of the PCR product.

## FUTURE EXPERIMENTS

The most important experiment to be performed is to use PCR to amplify the *lacI* gene. After amplification, the PCR products should be sequenced and compared with the wild type gene and the kanamycin resistance gene from plasmid pACYC177. An alternate approach is to performing a southern blot on the PCR products using the kanamycin cassette as a probe. Both approaches can give evidence to show whether or not the partial deletion of *lacI* was successful. Another important question to answer next is where the kanamycin cassette was inserted.

## ACKNOWLEDGEMENTS

I would like to thank Dr. W. D. Ramey for invaluable discussions on practical and theoretical aspects of the experiment, and Jennifer Sibley and Manpreet Cheema for assistance in the laboratory and support.

## REFERENCES

1. **Blattner, F.R., J.D. Glasner, and C.D. Herring.** 2003. "Gene replacement without selection: regulated suppression of amber mutations in *Escherichia coli*" *Gene* 331:153-163
2. **Chan, V., L.F. Dreolini, K.A. Flintoff, S.J. Lloyd, and A.A. Mattenley.** 2002. "The effects of glycerol, glucose, galactose, lactose and glucose with galactose on induction of  $\beta$ -galactosidase in *Escherichia coli*", *J.Exp. Microbiol. Immuno.* 2:130-137
3. **Datsenko, K. A. and B.L. Wanner.** 2000. "One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products" *Proc. Nat. Acad. Sci.* 97, 6640-66
4. **Devore, J.L.** 2000. *Probability and Statistics fro Engineering and the Sciences.* 5<sup>th</sup> ed. Pacific Grove, CA, U.S.A. Brooks/Cole
5. **Fermentas Lif Sciences.** 2004. pACYC177;description and restriction map. [<http://www.fermentas.com/techinfo/nucleicacids/mappacyc177.htm>];[01-11-05].

6. **Jaeger, A., P. Sims, R. Sidsworth, and N. Tint**, 2004. "Initial stages in creating a *lacI* knockout in *Escherichia coli* C28 using the lambda red recombinase system", *J. Exp. Microbiol. Immunol.* 5:65-71
7. **Markiewicz, P., L. G. Kleina, C. Cruz, S. Ehret and J. H. Miller**. 1994. "Genetic Studies of the *lac* Repressor. XIV. Analysis of 4000 Altered *Escherichia coli lac* Repressors Reveals Essential and Non-essential Residues, as well as "Spacers" which do not Require a Specific Sequence." *J. Molec Biol.* **240**, 421-433.
8. **Pearson Education Inc.** 2004 *Lac Operon: The Effects of Lactose on the Lac Operon*.  
[[http://www.phschool.com/science/biology\\_place/biocoach/lacoperon/effect.htm](http://www.phschool.com/science/biology_place/biocoach/lacoperon/effect.htm)][01-11-05]
9. **Ramey, W.D.** 2002. Effect of Glucose, Lactose and Sucrose on the Induction of  $\beta$ -Galactosidase. Microbiology 421 laboratory manual. University of British Columbia, Vancouver, B.C.
10. **Snyder, L., W. Champress.** 2003. *Molecular genetics of bacteria*. 2<sup>th</sup> ed. Washington, D.C, USA. ASM Press