

## Initial Stages in Creating a *lacI* Knockout in *Escherichia coli* C29 Using the Lambda Red Recombinase System

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**An attempt was made in this study to knockout the *lacI* gene in *Escherichia coli* C29 cells. This multi-step process, involving PCR and electroporation, was successful in many areas. The initial transformation of the C29 cells with the plasmid pKD46 using electroporation successfully conferred ampicillin resistance. The pKD46 plasmid introduced at this stage contains the lambda-red recombinase genes as well as an ampicillin resistance cassette. Primer design and subsequent amplification of the promoter-less kanamycin resistance gene from the pACYC177 plasmid, preceded the second electroporation of the C29 cells harboring pKD46. The PCR product was thought to be designed such that the flanking ends were homologous to the termini of the *lacI* gene. However, it was discovered that an upstream DNA binding domain remained and was fused to the kanamycin resistance cassette. Introduction of the linear DNA PCR product into the C29 cells resulted in kanamycin resistance. This result indicates successful integration of the PCR product into the genome of the C29 cells, for only under an endogenous promoter would expression of the kanamycin gene be observed. Supporting this observation is the activation of  $\beta$ -galactosidase ( $\beta$ -gal) in the absence of IPTG, for the cells expressed a blue phenotype when grown in LB/X-gal solid media. The blue phenotype was less intense than expected, suggesting only partial repression of the *lac* operon as a result of the DNA binding domain present in the fusion protein. When transformants were tested using an ONPG assay, higher basal levels of  $\beta$ -gal activity was observed when compared to control cells, yet both samples were equally sensitive to IPTG.**

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The *lac* operon is a well studied operon in *Escherichia coli*. It consists of a set of three structural genes whose expression is under the control of three regulatory genes. The metabolism of lactose requires two of the structural genes, Z and Y, which encode for the enzymes  $\beta$ -galactosidase and a permease respectively (3).  $\beta$ -galactosidase is responsible for the cleavage of lactose into galactose and glucose, whereas the permease transports the lactose into the cell (3). The third gene, gene A, encodes for the enzyme transacetylase, but is not needed for lactose metabolism (3). The regulatory genes consist of a repressor (I) the promoter (P), and the operator (O). The repressor (I) has two binding sites: a DNA binding site that binds at the operator, and an allosteric binding site that binds to lactose and other analogues of lactose, such as IPTG (3).

In order for the *lac* enzymes to be expressed, two specific conditions must be met. The first condition is glucose must absent in the surrounding environment and the second condition is lactose must be present in the environment (3). When lactose or lactose analogues such as IPTG are present, they allosterically bind to the repressor protein and change the shape of the repressor so that it is unable to bind to the operator; therefore, the structural genes Z, Y, and A are transcribed (3). When the two conditions are not met, the repressor binds to the operator and prevents binding of RNA polymerase.

Having an *E. coli* strain that constitutively expresses  $\beta$ -galactosidase would be beneficial. Such a strain would neither require IPTG for induction, nor the incubation times necessary for sufficient production of  $\beta$ -galactosidase required for assays. To create this mutant strain, a newly developed system for gene deletion could be employed. The  $\lambda$  Red recombination technology has been used in *E. coli* and *salmonella* for easy PCR mediated deletion mutations (6, 8). The system has three major proteins involved in the process (7). The first protein is the  $\lambda$  exonuclease, which digests the 5' end of the dsDNA in the host's chromosome (7). The second protein is a  $\beta$  protein, which binds to the bacterial RecBCD enzyme and inhibits its activity (7). The third major protein involved is a  $\gamma$  protein. It binds to ssDNA and encourages annealing (7).

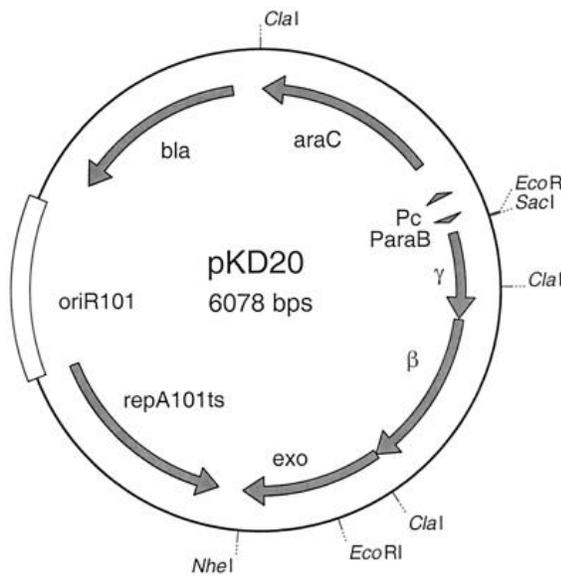
A selectable antibiotic resistance gene is created by PCR using PCR primers that are homologous to the targeted gene (6, 7, 8). The  $\lambda$  Red technology promotes specific gene replacement by electroporating this linear DNA into *E. coli* (6, 7). The host RecBCD exonuclease activity is then inactivated by the  $\beta$  protein, therefore, enabling efficient

recombination with the PCR created linear DNA (6, 7). The colonies with the resistance gene can easily be selected for (6, 8).

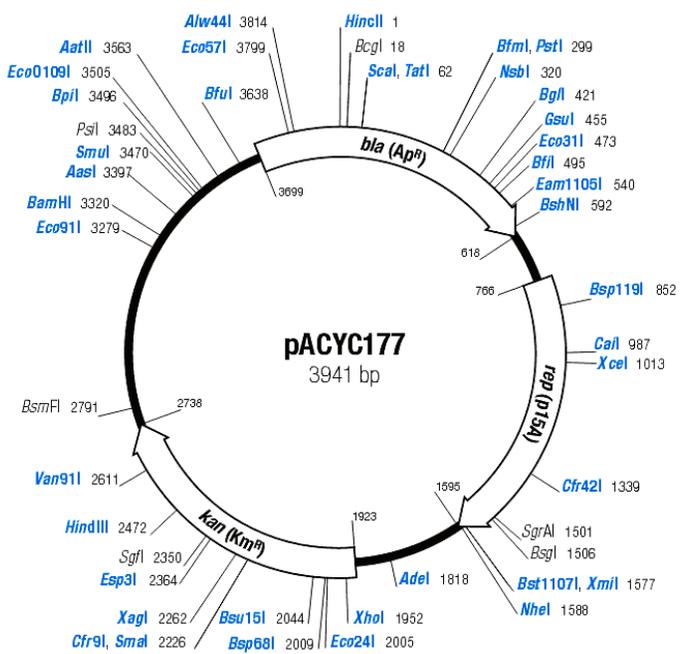
Here we describe a procedure and attempt to specifically knock out the *lacI* gene in the *lac* operon using the  $\lambda$  Red system. *LacI* will be replaced with a selectable antibiotic resistance gene generated by PCR using primers with 36 nt extensions. This will allow for constitutive expression of the *lac* operon without depending on the presence or absence of glucose and lactose. Also, the presence of lactose or lactose analogues such as IPTG should have no effect on the expression or amounts of  $\beta$ -galactosidase produced.

### MATERIALS AND METHODS

**Plasmids.** Plasmid pKD46 is 6329 bp, and contains the *araC-P<sub>araB</sub>*,  $\gamma$   $\beta$  *exo tL3* regions of  $\lambda$  phage DNA (Fig. 1). Plasmid pACYC177 (gift from Ivan Sadowski, Dept. of Biochemistry, UBC) is 3941 bp, containing *rep*, *kan*, *bla*, conferring plasmid replication (*rep*), kanamycin resistance (*kan<sup>R</sup>*), and ampicillin resistance (*amp<sup>R</sup>*), respectively (Fig. 2). Plasmid pET30b contains *kan*, conferring kanamycin resistance. Plasmids were isolated from overnight broth cultures using a standard alkaline lysis protocol. 3  $\mu$ L of isolated pKD46 was incubated in 50  $\mu$ L 10x buffer, 0.5  $\mu$ L BSA, and 1  $\mu$ L of either *SstI* or *NheI*; digested samples were electrophoresed on a 0.7% agarose gel in TAE buffer, at 100 V.



**Figure 1.** Restriction Digest Map of plasmid pKD20. Plasmid pKD46 is identical to pKD20, except for the addition of  $\lambda$  tL3 terminator in pKD46.



**Figure 2.** Restriction Digest Map of plasmid pACYC177.

**Media.** Broth cultures of *E. coli* C29 cells (obtained from MICB 421 laboratory) were grown at 37°C with shaking in Luria Broth (pH 6.9); LB broth is comprised of 1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl, 15% 10 M NaOH. The SOB media used to prepare competent *E. coli* C29 cells is composed of 10 g tryptone, 2.5 g yeast extract, 0.2 g NaCl, 0.093 g KCl, 10 M MgCl<sub>2</sub> and 10 M MgSO<sub>4</sub> in 500 ml. The modified SOB media used to prepare competent Amp<sup>R</sup> *E. coli* C29 cells for the second transformation contained 10% 1 M L-arabinose, in addition to the standard components. Z buffer used for the  $\beta$ -galactosidase assay is comprised of Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O (0.06 M), Na<sub>2</sub>HPO<sub>4</sub>·H<sub>2</sub>O (0.06 M), KCl (0.01 M), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.001 M) and  $\beta$ -mercaptoethanol (0.05 M) and brought to pH 7.0.

**Cells.** *Escherichia coli* BW2511 cells (kindly provided by Dr. Brett Finlay's lab) harboring plasmid pKD46 were grown in Luria Broth containing 100  $\mu$ g/ml ampicillin; *E. coli* DH5a cells (Invitrogen) harboring plasmid pACYC177 were grown in Luria Broth containing a final working concentration 50  $\mu$ g/ml kanamycin. All broth cultures were incubated overnight at 37°C with aeration. Amp<sup>R</sup> cells (transformed *E. coli* C29 cells harboring the plasmid pKD46) were selected for 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 or pET30b) were selected for on solid LB plates containing a final working concentration of 50  $\mu$ g/ml kanamycin.

**Primers and PCR.** The sequence of the forward primer used is 5'-ATGGCGAGCTGAATTACATTCCTCAACCCTGGTGGCATGCGTTGTGCGGGAAGATGCG-3'; the sequence of the reverse primer used is 5'-TCACTGCCCGCTTCCAGTCGGGAAACCTGTCGTGCGTGATCTGATCCTTCAACTC-3'. Forward and reverse primers were designed to contain 36 nucleotides of the *E. coli* K12 *lacI* gene as well as 20 nucleotides flanking the kanamycin resistance cassette contained in the pACYC177 plasmid. The forward primer was thought to contain the first 36 nucleotides of *lacI* and the first 20 nucleotides of the KanL flanking region. However, in hindsight the priming sequence matched the region of *lacI* at position 154 of the gene (2), leaving an in-frame segment upstream from the kanamycin cassette. The reverse primer contained nucleotides that are complimentary to the last 20 nucleotides of the KanR flanking region and the last 36 nucleotides of *lacI*. The primer sequences were constructed using NetPrimer URL, BLAST against the *E. coli* K12 total genome to determine specificity and then synthesized by the Nucleic

Acid Protein Synthesis Unit (NAPS, UBC). The dried primer pellet was resuspended in sterile distilled water to a concentration of 30  $\mu\text{M}$ , as determined by  $\text{OD}_{260}$  values.

Amplification of the kanamycin resistance cassette-*lacI* construct was conducted using 12 samples of 0.5  $\mu\text{L}$  Platinum *pf*x DNA Polymerase (Invitrogen Life Technologies, city), 5  $\mu\text{L}$  of each primer (30  $\mu\text{M}$ ) and 1  $\mu\text{L}$  of pACYC177 template DNA. A temperature gradient of 50-70°C was conducted to determine the optimum annealing temperature; PCR reaction was 120 seconds at 95°C, 45 seconds at 95°C, 45 seconds at either 50.0°, 50.5°, 51.9°, 54.0°, 56.4°, 58.8°, 61.2°, 63.6°, 65.9°, 68.1°, 69.5° or 70.0°C, followed by 90 seconds at 72°C and 10 minutes at 72°C; steps 2-4 were repeated for 30 cycles.

PCR products were electrophoresed on 1% agarose gel at 100 V; after confirmation of PCR products of expected size, DNA was purified from other PCR components using a standard phenol:chloroform extraction and ethanol precipitation.

**Preparation of Electrocompetent Cells and Transformations.** *First transformation:* *Escherichia coli* C29 cells were incubated in 25 ml SOB media at 30°C with aeration, until an  $\text{OD}_{600}$  of 0.6 was reached; the 25 ml broth culture was then centrifuged at 6,000  $\times g$  for 15 minutes. After discarding the supernatant, the pellet was washed in 5 ml 10% cold glycerol, and centrifuged at 6,000  $\times g$  for 15 minutes. The 10% cold glycerol wash and centrifugation was repeated twice, for a total of 3 washes; the final *E. coli* C29 pellet was resuspended in 100  $\mu\text{L}$  of cold sterile distilled water, and stored on ice. 50  $\mu\text{L}$  of competent *E. coli* C29 cells and 1  $\mu\text{L}$  of isolated pKD46 were combined within the BIO-RAD 0.2 cm cuvette (catalogue #165-2086) and using the BIO-RAD MicroPulser, were pulsed at 2.5 kV for 1 second (program EC2), in accordance with manufacturer's instructions. In addition, another 50  $\mu\text{L}$  of competent *E. coli* C29 cells were electroporated in the presence of 1  $\mu\text{L}$  isolated pBlueScript Sk<sup>+</sup> (pBS Sk<sup>+</sup>) as a positive control. Immediately following electroporation, the cells were incubated in 1 ml SOB media at 37°C for 1.5 hours. Successful transformants were selected by spreading 200  $\mu\text{L}$  of each electroporation on solid LB plates that contain a final working concentration of 100  $\mu\text{g}/\text{ml}$  ampicillin; all plates were incubated at 30°C overnight or until growth was observed.

*Second transformation:* Two Erlenmeyer flasks containing Amp<sup>R</sup> *E. coli* C29 cells in 25 ml modified SOB media with 100  $\mu\text{g}/\text{ml}$  ampicillin, were incubated at 30°C with aeration until an  $\text{OD}_{600}$  of 0.6 was reached. The 10% cold glycerol washes and centrifugation steps previously described were repeated, and the resultant pellets were each resuspended in 100  $\mu\text{L}$  of cold sterile distilled water. 50  $\mu\text{L}$  of electrocompetent Amp<sup>R</sup> *E. coli* C29 cells were combined with 1  $\mu\text{L}$  PCR product (30  $\mu\text{M}$ ) within the BIO-RAD 0.2 cm cuvette (catalogue #165-2086); in accordance with manufacturer's instructions, using the BIO-RAD MicroPulser, cells were pulsed at 2.5 kV for 1 second (program EC2). Three additional independent transformations were completed, using 50  $\mu\text{L}$  Amp<sup>R</sup> *E. coli* C29 cells and either 5  $\mu\text{L}$  PCR product (30  $\mu\text{M}$ ), 1  $\mu\text{L}$  pET30b (positive control), or 1  $\mu\text{L}$  sterile distilled water (negative control). Successful transformants were selected by spreading 200  $\mu\text{L}$  of each electroporation on solid LB plates that contain a final working concentration of 50  $\mu\text{g}/\text{ml}$  kanamycin; all plates were incubated at 30°C overnight or until growth was observed. Colonies that grew on the LB-kanamycin plates were streaked onto solid LB plates containing 1  $\mu\text{L}/\text{ml}$  X-gal, in addition to 50  $\mu\text{g}/\text{ml}$  kanamycin. The plates were incubated at 37°C overnight; 2 colonies that appeared blue after the overnight incubation were further colony purified by streaking a second X-gal, kanamycin, LB plate and incubating the second set of plates overnight at 37°C; the colony purification was repeated for 2 colonies that appeared white after transformation with positive control pET30b.

**$\beta$ -galactosidase Assay.**  $\beta$ -galactosidase productivity and activity was assayed by a combination of protocols outlined by J. Miller (5) and W.D. Ramey (9). A single colony from each plate in the second set of X-gal, kanamycin, LB plates was inoculated into 5 ml Luria Broth containing kanamycin (50  $\mu\text{g}/\text{ml}$ ), and incubated with aeration overnight at 37°C. One overnight control culture of Amp<sup>R</sup>, Kan<sup>R</sup> (pET30b) *E. coli* C29 cells was used to inoculate two 10 ml cultures of LB containing kanamycin (50  $\mu\text{g}/\text{ml}$ ); 0.1 M IPTG was added to one of 10 ml cultures with a final concentration of 1 mM IPTG. One overnight culture of Amp<sup>R</sup>, Kan<sup>R</sup> (PCR product) *E. coli* C29 cells was used to inoculate two 10 ml cultures of LB containing kanamycin (50  $\mu\text{g}/\text{ml}$ ); 0.1 M IPTG was added to one of 10 ml cultures with a final concentration of 1 mM IPTG. All four 10 ml cultures were incubated with aeration at 37°C for 4 hours. 3 ml of each culture was mixed with 200  $\mu\text{L}$  toluene, followed by thorough vortexing. After allowing the toluene to separate into a distinct phase, 0.4 ml of each cell culture was mixed with 1.2 ml Z buffer and 0.2 ml ONPG (5 mM); the reaction was carried out in a 30°C water bath, and stopped with the addition of 2 ml Na<sub>2</sub>CO<sub>3</sub> (0.6 M). The resulting optical density was measured at  $A_{420\text{nm}}$ .

## RESULTS

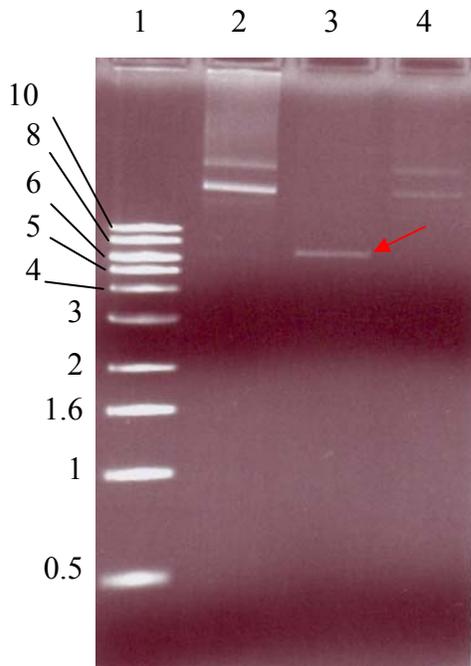
Plasmid pKD46 was singly digested with either *Sst*I or *Nhe*I; the *Sst*I digestion yielded a band at 6 kb, the expected plasmid size. Digestion with *Nhe*I resulted in a banding pattern identical to that of the undigested plasmid, upon gel electrophoresis (Fig.3).

The transformation of *E. coli* C29 cells with either pKD46 or the control expression vector pBS Sk<sup>+</sup> resulted in extensive growth on solid LB-ampicillin plates, indicating a successful transformation.

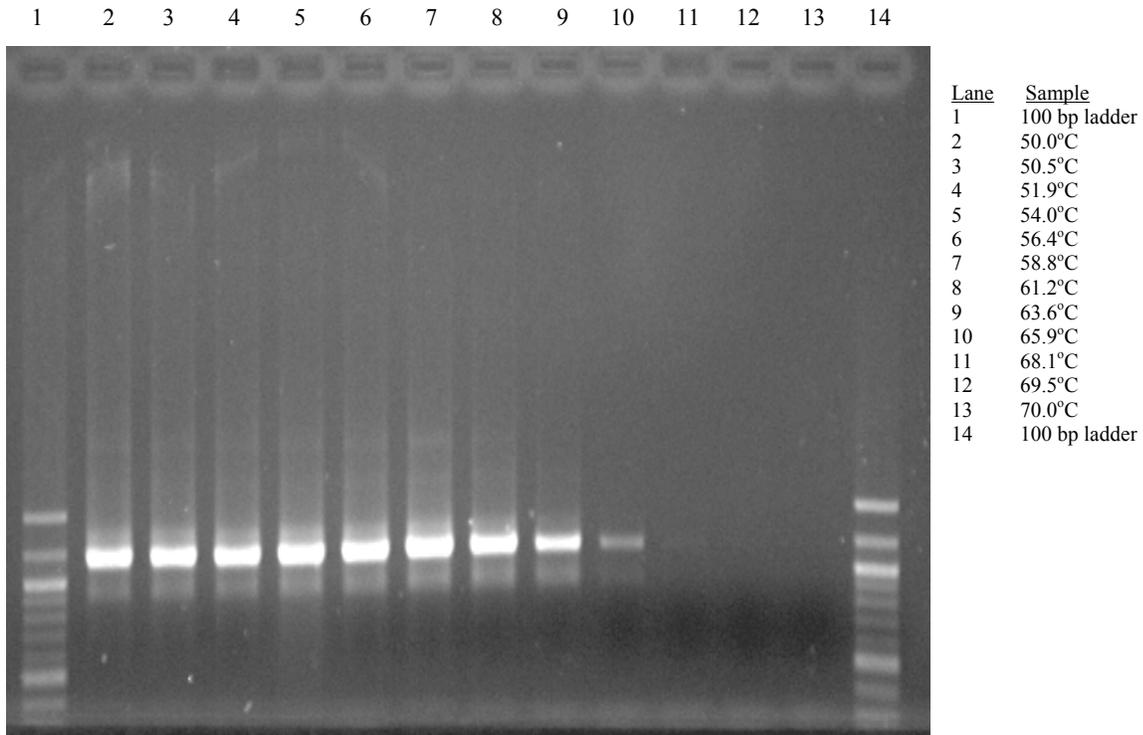
The gradient PCR was performed using a range of temperatures between 50.0° and 70.0°C. The optimal annealing temperature was shown to be 58.8°C (Fig. 4), as measured by band thickness and brightness following electrophoresis.

Amp<sup>R</sup> *E. coli* C29 cells harboring plasmid pKD46 were transformed with the PCR DNA product or plasmid pET30b and plated on LB-Kan plates. Adequate growth was observed on all plates, and 60 isolated colonies were plated on X-gal-Kan-LB plates. The control plates appeared to support the growth of consistently white colonies, while colonies of transformants harboring the PCR product appeared blue in the center, and were surrounded by a ring of white growth.

Broth cultures of Amp<sup>R</sup>Kan<sup>R</sup> *E. coli* C29 and pET30b<sup>+</sup> *E. coli* C29 were incubated in the presence or absence of IPTG; the resulting  $\beta$ -galactosidase levels were assayed as previously described. Both strains of transformants showed an increased level of  $\beta$ -galactosidase production in the presence of IPTG (Fig. 5).

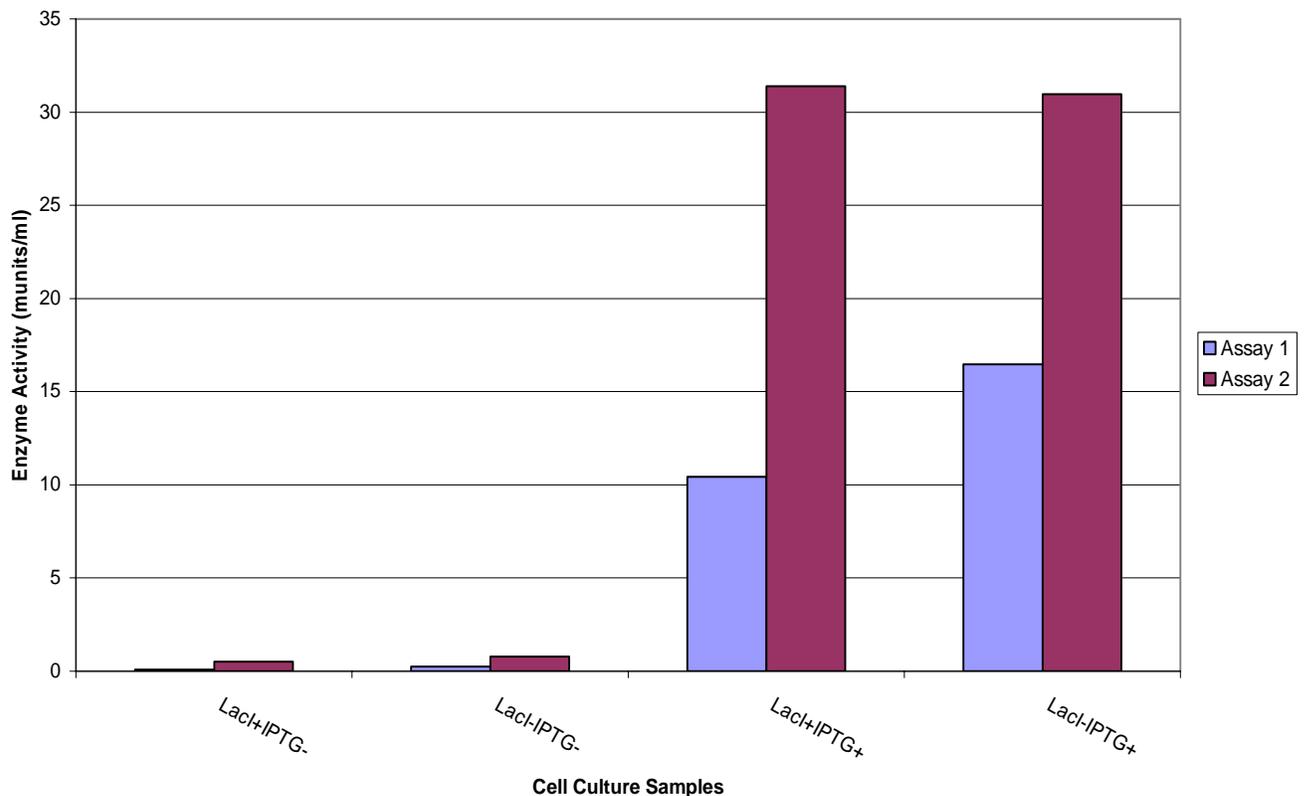


**Figure 3.** Restriction enzyme digests of pKD46. Agarose gel (1%) was stained with ethidium bromide and visualized using an ultraviolet light source. The red arrow indicates the appropriate size band for the linearized pKD46 plasmid.



**Figure 4.** Gel Electrophoresis of PCR DNA products. Products resulting from gradient PCR amplification of *kan<sup>R</sup>* cassette, using pACYC177 as template DNA and the designed primers.

Fig. 5  $\beta$ -galactosidase production, in the presence or absence of IPTG, of *lacI*<sup>-</sup> and *lacI*<sup>+</sup> *E. coli* C29



## DISCUSSION

The lambda ( $\lambda$ ) red system used for gene disruption in *E. coli* C29 is based on the system developed by Datsenko and Wanner (1) for *E. coli* K12, and the system used in several other labs for the study of EPEC and EHEC (6). The lambda red system offers the opportunity for efficient, simple gene disruption through homologous recombination. Initially, gene disruption requires the activation of recombinase genes, which in this case are present on the pKD46 plasmid. To ensure the plasmid we were provided with was in fact pKD46, a restriction digest and gel electrophoresis was performed with enzymes *SstI*, *NheI* to look for predicted banding patterns. *SstI* and *NheI* both contain single cut sites within pKD46, therefore, we expected a single band in both lanes of the agarose gel (1). The banding patterns produced by *SstI* following the digest, confirmed that we had a plasmid of size 6 kb, which was expected. While *SstI* produced the expected results, *NheI* did not. This digest produced an identical banding pattern to that of the undigested plasmid, suggesting that either the *NheI* cut site was missing in the pKD46 plasmid, or the restriction enzyme was inactive. A missing cut site could be explained by a mutation in the plasmid which could alter the DNA sequence recognized by the *NheI* restriction enzyme. Although the pKD46 plasmid may contain a mutation at the *NheI* cut site, this should not impact the plasmid's gene products since the *NheI* specific sequence is located downstream from the exonuclease open reading frame (1).

The transformation of *E. coli* C29 using the pKD46 plasmid was highly successful. The protocol for providing the recombinase genes, blocking recBCD, and conferring ampicillin resistance on a single pKD46 plasmid was expected to produce transformants with resistance to ampicillin (1); success was measured by the number, and purity of transformants obtained. Expected results were obtained, as ampicillin-LB plates supported the growth of numerous colonies.

Perhaps the most important step in the lambda red system is the production of the PCR products to be used in homologous recombination. Maximizing the purity and quantity of PCR product is likely to improve the results of the gene disruption significantly. Different temperatures were tested to determine the optimum annealing

temperatures for this specific PCR reaction, and it was discovered that our primers annealed to the pACYC177 template plasmid optimally at 58.8 °C. This annealing temperature produces a large amount of PCR product, which was simple to visualize by agarose gel electrophoresis. By maximizing the PCR reaction, we were able to produce more than enough product (in this case, the kanamycin resistance gene) to allow for optimal conditions for the second transformation.

The second transformation was to produce our end products, amp<sup>R</sup>kan<sup>R</sup>lacI<sup>-</sup> *E.coli* C29. Expectations were to see colonies of kanamycin resistant cells with a disrupted *lacI* gene, and therefore, constitutive expression of  $\beta$ -galactosidase ( $\beta$ -gal). Plating of transformants on plates containing X-gal, and kanamycin produced numerous colonies with kanamycin resistant, and presumably constitutive expression of  $\beta$ -gal, based on their blue colour. Although the colonies were clearly kanamycin resistant, their colour was not a “brilliant blue”, but rather a blue surrounded by a milky ring, indicating lower levels of  $\beta$ -gal production. Nevertheless, the X-gal / Kanamycin plates appear to have selected for those transformants in which the lambda red recombination system was successful. The high number of colonies, and their phenotypic similarity, demonstrates the ability of the lambda red gene disruption system to produce efficient, identical gene knockouts in a high number of cells.

To further test the extent of efficiency and success of recombination in the amp<sup>R</sup>kan<sup>R</sup> *E. coli* C29 transformants, a set of simple ONPG assays was performed. Since our cell line was believed to be missing the *lacI* gene, it was expected that adding IPTG would have no impact on the levels of  $\beta$ -gal production; however, as is demonstrated in fig 4, this was not the case with our transformants. Although our knockouts did produce slightly higher basal levels of  $\beta$ -gal than the control cell line, it is clear that IPTG still induces the production of  $\beta$ -gal in the transformed *E. coli* C29. These results were particularly unexpected because it was assumed that the creation of kanamycin resistant colonies would depend on the elimination of the *lacI* gene. However, as mentioned above, in-frame fusion of the kanamycin resistance cassette with the upstream region of *lacI* would result in a gene product that would not only confer kanamycin resistance, but still bind the operator of the *lac* operon. The recognition of the operator is made possible through the N-terminal DNA binding domain now present in the translated protein (4). If this fusion indeed occurred, then this may account for the results observed in both the ONPG and X-gal assays. Repression of the *lac* operon would still occur through the DNA binding ability of the fusion protein (though it appears only partial repression was achieved). This partial repression would explain the positive results (low-intensity blue coloration) of the X-gal assay. What is interesting is that the addition of IPTG still resulted in the increased expression of the *lac* operon; this result is interesting because of the reported binding region for IPTG is found in the C-terminus of LacI (Ramey, W. personal communication). Thus, these results may suggest that IPTG has some ability to recognize the N-terminal portion of LacI.

It should be noted that the start codon of the *lacI* gene is a GTG (2). This was the reason for having the upstream DNA binding domain of *lacI* remaining in the chromosome. Most genes possess ATG start codons, and open reading frames of the *lacI* gene were searched using ATG as a probe. Unfortunately, time constraints resulted in this oversight. However, what appears to be a mistake in the molecular biology of the experiment may in fact open new areas of research into the DNA binding domain of the LacI repressor.

## FUTURE EXPERIMENTS

Perhaps the most immediately applicable experiments which could be conducted at this point include a second PCR and Southern Blot analyses, to allow a more precise determination of the genetic makeup of the kanamycin resistant transformants. By using a primer sequences specific to an internal region of *lacI* (distal to the DNA binding domain) and total genomic DNA of the kan<sup>R</sup> mutants as template, production of a PCR product would indicate that the *lacI* gene is still present. Also, a similar PCR experiment now using a primer specific to the DNA binding domain may indicate if this region is still present. Alternatively, a Southern Blot could be performed on total genomic DNA of kan<sup>R</sup> transformants, using a probe specific to the *lacI* gene. Positive results in the Southern blot experiment would indicate the persistence of the *lacI* gene in the genome of the target cells.

In light of these results, it is recommended that the procedure be repeated using the already purified PCR products and pACYC177 as the control plasmid.

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