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Assessment of Targeting the Lambda Red Recombinase System to the Intended Disruption of the *lac*I gene in *Escherichia coli* C29

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An attempt was made to knockout the *Escherichia coli* C29 *lac*I gene with a kanamycin resistance cassette isolated from the plasmid pACYC177. This process used the lambda-red recombinase system to allow for homologous recombination, and subsequent insertion of the kanamycin cassette into the *lac*I gene. The *E. coli* C29 cells were initially transformed with pKD46, which contains the lambda-red recombinase genes, along with an ampicillin resistance cassette. The resulting ampicillin transformants were transformed with a linear DNA PCR product, the kanamycin cassette with flanking ends that were homologous to the termini of the *lac*I gene. If the *lac*I gene was successfully knocked out, then the β-galactosidase gene should be constitutively expressed. However, when the kanamycin resistant cells were grown on media containing X-gal, the colonies had a faint blue phenotype. The blue phenotype was not the intense blue phenotype that was expected in cells with high levels of β-galactosidase production. When these transformants were tested using an ONPG assay, the cells showed an increase in β-galactosidase activity in the presence of IPTG, at the same levels that were seen the original C29 cells.

The lac operon is one of the most extensively investigated operon system within *Escherichia coli.* The *lac* operon consists of one regulatory gene (*lac*I) and three structural genes (*lacZ*, *lacY*, and *lacA*) (4). The *lac*I gene codes for the repressor of the *lac* operon. The *lacZ* gene codes for β-galactosidase, which is primarily responsible for the hydrolysis of lactose into galactose and glucose. The *lac*Y gene codes for permease, which increases permeability of the cell to β-galactosides, and *lac*A gene encodes a transacetylase (4). During normal growth on a glucose-based medium, the LacI repressor is bound to the operator region of the *lac* operon, preventing transcription. However, in the presence of an inducer of the *lac* operon, such as IPTG, the repressor protein binds the inducer and is incapable of interacting with the operator region of the operon.

Disruption of the *lac*I gene would result in having an *E. coli* strain that constitutively expresses β-galactosidase. The creation of such as strain would allow for β-galactosidase production without IPTG induction, which is more economical for β-galactosidase production required for assays. The deletion of the *lac*I gene was created using the lambda (λ) red recombination technology and homologous recombination. The lambda red recombination system has three major proteins: the λ exonuclease, which degrades the 5' end of dsDNA of the host's chromosome; the β protein involved in the binding to the RecBCD enzyme and inhibiting its activity; the γ protein that promotes ssDNA annealing (6).

Homologous recombination is targeted using a selectable marker, such as an antibiotic resistance gene cassette, flanked by sequences at the termini of the *lac*I gene (6). Efficiency of recombination is enabled by the inactivation of host cellular RecBCD activity and allowing the linear dsDNA to recombined within the chromosome (6). Successful recombination into the *lac*I gene should result in the constitutive expression of β-galactosidase, and the loss of induction via allactose, IPTG or similar lactose analogs.

MATERIALS AND METHODS

Plasmids. Plasmid pACYC177 is 3941 bp, containing *rep* (plasmid replication), *kan* (kanamycin resistance), and *bla* (ampicillin resistance) (8). Plasmid pKD46 is 6329 bp, and contains the genes required for the lambda red recombination technology (1). Plasmids were isolated from *E. coli* cells using the Qiagen QIAamp Tissue Kit.

Bacterial Strains. *Escherichia coli* DH5α cells harboring plasmid pACYC177 were grown in Luria Broth containing a final working concentration of 50μg/ml kanamycin; *Escherichia coli* BW2511 harboring plasmid pKD46 were grown in Luria Broth containing a final working concentration of 100μg/ml ampicillin. Luria Broth (LB) medium (pH 6.9, 10g tryptone, 5g yeast extract, 10g NaCl) cultures were incubated overnight at 37°C with shaking. *E. coli* C29 cells transformed with plasmid pKD46 were selected on solid LB-plates containing a final concentration of 100μg/ml ampicillin. *E. coli* C29 cells harboring plasmid pKD46 transformed with kanamycin PCR product were selected for on solid LB plates containing a final working concentration of 50μg/ml kanamycin. Competent *E. coli* C29 cells were prepared using SOB media composed of 10g tryptone, 2.5g yeast extract, 0.2g NaCl, 0.093g KCl, 10M $MgCl_2$ and 10M $MgSO_4$ in 500ml

Primers and PCR. The sequence of the forward primer used was5'AGGGTGGTGAATGTGAAACCAGTAACGTTATACGATT GCGTTGTCGGGAAGATGCG-3'; the sequence of the reverse primer used is 5'-TCACTGCCCGCTTTCCAGTCGGGAAAC CTGTCGTGCGTGATCTGATCCTTCAACTC-3' (gift from Jaeger *et al*) (3). Jaeger *et al* designed the reverse primer to contain the last 36 nucleotides of the *E. coli* K12 *lac*I gene as well as the last 20 nucleotides flanking the kanamycin resistance cassette contained in the pACYC177 plasmid (2, 10). The forward primer was designed by

hand to contain 12 nucleotides upstream from the *lac*I gene, 24 nucleotides of *lac*I, and first 20 nucleotides flanking the kanamycin resistance cassette contained in the pACYC177 plasmid. The forward primer was synthesized by the Nucleic Acid Protein Synthesis Unit (NAPS, UBC). The dried pellet was resuspended in sterile distilled water to a concentration of 168µM. The kanamycin resistance cassette-*lac*I construct was amplified with the following reagents: 5.0 µl 10X Platinum *pfx* amplifying buffer, 1.5 µl dNTP mix, 1.0 µl 50mM $MgSO_4$, 2.0 µl of each primer, and 2.0 µl of pACYC177 template DNA, 0.4 µl Platinum *pfx* DNA polymerase (Invitrogen Life Technologies), and 36.1 µl of dH_2O to a final volume of 50 µl. A temperature gradient of 42-72°C was conducted to determine the optimum annealing temperature. The PCR reaction conditions used were an initial denaturation of 4 minutes at 95°C, 40 cycles of 1 minute at 95°C, 1 minute at 42°C, 42.7°C, 44.8°C, 48.1°C, 51.6°C, 55.2°C, 58.8°C, 62.3°C, 65.9°C, 69.2°C, 71.3°C or 72.0°C, followed by 1 minute at 72°C and a final extension of10 minutes at 72°C.

Preparation of Electrocompetent Cells and Transformations. One liter of *Escherichia coli* C29 cells in LB broth were incubated at 37°C with aeration, until an OD_{600} of 0.70 was reached. The broth culture of the *Escherichia coli* C29 cells was then centrifuged at 6,000 x g for 15 minutes. After discarding the supernatant, the pellet was resuspended in 500 ml of 10% glycerol, and centrifuged at 6,000 x g for 15 minutes. The subsequent supernatant was discarded, and the pellet was resuspended in 250 ml of 10% glycerol, and centrifuged at 6,000 x g for 15 minutes. Again, the supernatant was discarded, and the pellet was resuspended in 20 ml of 10% glycerol, and centrifuged at 6,000 x g for 15 minutes. The final *Escherichia coli* C29 pellet was resuspended in 2.0 ml of 10% glycerol. The cells were flash frozen on dry-ice and stored at -80°C until further use.

First Transformation. 50 µL of competent *E. coli* C29 cells and 1 µL of isolated pKD46 were combined within the BIO-RAD 0.2 cm cuvette (catalogue #165-2086). Using the BIO-RAD MicroPulser, the *E. coli* C29 cells and pKD46 were pulsed at 2.5 kV for 1 second (program EC2). In addition, another 50 µL of competent *E. coli* C29 cells were electroporated in the presence of 1 µL sterile water as a negative control. Immediately following electroporation, the cells were incubated in 500 µL SOB media at 37°C with aeration for 1.5 hours. Successful transformants were selected by spreading 200 µl of each electroporation on solid LB plates with a final working concentration of 100 µg/ml ampicillin. Plates were incubated at 37°C overnight.

Second transformation. One liter of ampicillin resistant (Amp^R) *Escherichia coli* C29 cells in LB broth, with 100 µg/ml ampicillin, were incubated at 37°C with aeration until an OD_{600} of 0.70 was reached. Electrocompetent cells were prepared using the 10% cold glycerol washes and the centrifugation steps were repeated as previously described. 50 µL of electrocompetent Amp^R *E. coli* C29 cells were combined with 1 µl of PCR product within the BIO-RAD 0.2 cm cuvette (catalogue #165-2086). Using the BIO-RAD MicroPulser, cells were pulsed at 2.5 kV for 1 second (program EC2). Additional independent transformations were completed, using 50 µl Amp^R *E. coli* C29 cells and 5 µl PCR product, and 1 µl sterile distilled water as the negative control. Successful transformants were selected by spreading 200 µl of each electroporation on solid LB plates with a final concentration of 50 µg/ml kanamycin. Plates were incubated at 37°C overnight or until growth was observed. Colonies that grew on the LB-kanamycin plates were spotted onto solid LB-kanamycin plates containing 1 µl/ml X-gal, or LB-kanamycin plates containing 1 µl/ml X-gal and 1 mM isopropyl-β-D-Thiogalactopyranoside (IPTG). Plates were incubated at 37°C overnight.

β-galactosidase Assay. β-galactosidase productivity and activity was assayed using the protocols outlined by W.D. Ramey (7). Ten randomly selected colonies from LB-kanamycin X-gal plates were inoculated into 3 ml Luria Broth containing kanamycin (50 µg/ml), and 3 ml Luria Broth containing kanamycin (50 µg/ml) and IPTG (1 mM). The same ten colonies were also inoculated into 3 ml M9 broth containing kanamycin (50 µg/ml), and 3 ml M9 broth containing kanamycin (50 µg/ml) and IPTG (1 mM). Tubes were incubated with aeration at 37°C overnight. *E. coli* C29 cells (positive control) were inoculated into two 3 ml cultures of LB, and 3 ml of M9 broth with one tube of LB broth and M9 broth containing a final concentration of 1 mM IPTG. Each culture was mixed with 200 µl 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 Tris buffer and 0.2 ml o-nitrophenly-β-D-galactopyranoside (ONPG) (5 mM). The reaction was carried out in a 30°C water bath, and stopped with the addition of 2 ml Na_2CO_3 (0.6 M). The resulting optical density was measured at A_{420nm}.

RESULTS

Plasmid pKD46 was isolated from *E. coli* BW2511 cells using the Qiagen QIAamp Tissue Kit. Mini-prep and Maxi-prep QIAfilter kits isolated concentrations that were too low to be used in the experiment.

The transformation of *E. coli* C29 cells with pKD46 resulted in extensive growth on the LB-ampicillin plates, suggesting a successful transformation. The negative control, transformation of *E. coli* C29 cells with sterile water, resulted in no growth on LB-ampicillin plates.

Gradient PCR was performed using a range of annealing temperatures between 42°C-72°C to find the optimal annealing temperature. The optimal annealing temperature was 65.9°C as indicated by the brightness and thickness of the band (Fig. 1).

Ampicillin resistant *E. coli* C29 cells containing pKD46 were transformed with the kanamycin cassette PCR product. Growth was observed on all plates, and 64 isolated colonies were spotted onto LB plates containing both kanamycin and X-gal. The negative control plates, transformed with water, showed no growth on LB plates containing kanamycin. The 64 isolated colonies were also spotted onto LB plates containing kanamycin, X-gal, and IPTG. All colonies showed a faint blue color in both the presence and absence of IPTG.

Luria Broth and M9 broth cultures of ten randomly selected colonies of Amp^RKan^R *E. coli* C29 cells were grown in the presence and absence of IPTG. These cultures were used to assay the β-galactosidase levels as previously described above. The Amp^RKan^R *E. coli* C29 cells showed increased levels in β-galactosidase production in the presence of IPTG (Fig. 2).

DISCUSSION

The lambda (λ) red system utilizes homologous recombination in order to do a simple gene disruption. For this reason, it was important to verify that the pKD46 was the correct plasmid isolated. However, due to very low yields in the plasmid isolation, this verification step was not carried out. On the other hand, the transformation of *E. coli* C29 cells with pKD46 was very successful. Upon transformation with pKD46, the transformants were expected to confer ampicillin resistance, and this was supported by the number and

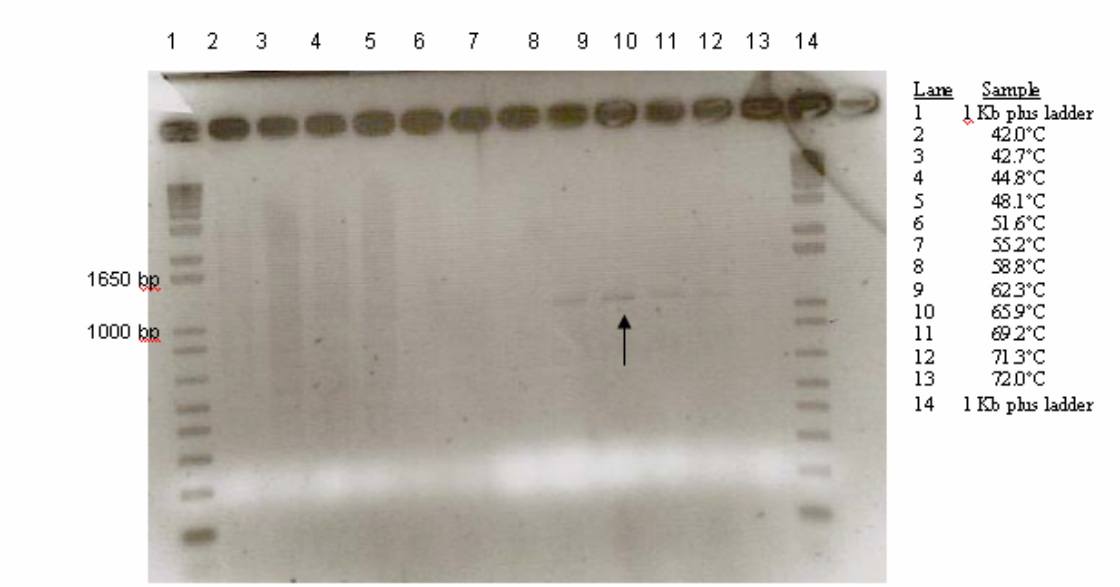

FIG. 1 Gel Electrophoresis of PCR DNA products. The product, kanamycin resistance cassette, is amplified using pACYC177 as the template and the designed primers. The band of the PCR product is pointed out by the arrow.

purity of transformants obtained on ampicillin-LB plates. Numerous colonies grow on all ampicillin-LB plates, and no growth was observed on the *E. coli* C29 cells transformed with water.

Since homologous recombination is one of the most important criteria for gene disruption using the λ red system, it is vital to obtain the correct PCR product. A temperature gradient was used to determine the optimum annealing temperature in order to maximize the purity of the PCR product. It was found that 65.9°C was the optimum annealing temperature as measured by the purity and quantity of the PCR product as seen via gel electrophoresis. The amplification of the kanamycin resistance cassette flanked by *lac*I provides the optimal conditions for homologous recombination into the *lac*I gene after the second transformation.

Transformation of the Amp^R *E. coli* C29 cells with the PCR product was to produce $amp^R kan^R lacI^-$ *E. coli* C29 transformants. With the disruption of the *lac*I gene, the β-galactosidase should be constitutively expressed within these cells. Upon plating these cells on kanamycin X-gal LB plates, all colonies showed a faint blue color, indicating a low level of β-galactosidase production. However, the high number of colonies that conferred kanamycin resistance suggests that the lambda red recombination system was successful at inserting the kanamycin gene cassette within the *E. coli* C29 chromosome.

To determine the efficiency and success of the recombination in the $amp^R kan^R$ *E. coli* C29 transformants, the β-galactosidase activity was measured using an ONPG assay. Ten randomly selected colonies were grown up in LB broth, as well as in M9 broth. Some of the colonies inoculated in the M9 broth did not grow, and could not be used in measuring the efficiency of the recombination. Since the homologous recombination should have disrupted the *lac*I gene, the addition of IPTG should have no impact on the production of β-galactosidase. However, as shown in Fig. 2, IPTG still induced the production of β-galactosidase in the kanamycin resistant isolates of *E. coli* C29 cells grown in LB broth, at the same level as seen in the control cells. The variation in the production of β-galactosidase in the kanamycin resistant isolates of *E. coli* C29 cells could be due to slight differences in growth conditions, experimental error, or even perhaps due to minor genetic differences in the kanamycin resistant isolates. These results suggest that homologous recombination was not successful at disrupting the *lac*I gene. But, if the creation of kanamycin resistant colonies is dependent on the insertion into the *E. coli* chromosome, this suggests that the insertion of the kanamycin resistance cassette occurred elsewhere within the genome.

Since the ONPG assay showed no constitutive expression of β-galactosidase, it suggests that the kanamycin resistance cassette is not inserting within the *lac*I gene. A similar anomaly was seen by Jaegar *et al,* with a different linear DNA, where the addition of IPTG still resulted in the increased expression of LacI (3). Since the kanamycin resistance cassette is located on a transposon, Tn903 (8), it is possible that the *E. coli* C29 genome also contains a transposon that has inverted repeats that are similar to that of Tn903. The amplified kanamycin cassette contained over 100bp of

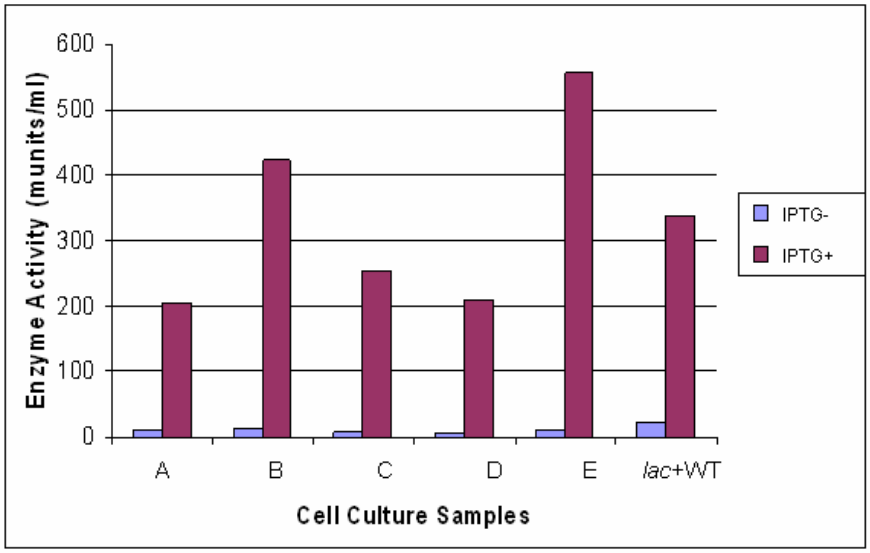

FIG. 2 Production of β-galactosidase in kanamycin resistant isolates of the *E. coli* C29 (A,B,C,D,E), and *lac*I+ WT *E. coli* C29 cells in the presence and absence of IPTG.

the Tn903 inverted repeats flanking both sides. These regions could provide potential homologous recombination sites and result in the insertion of the kanamycin resistant cassette at a different location within the genome. If another transposon is in the *E. coli* C29 genome, it is also possible that transposition events are also moving the kanamycin cassette.

FUTURE EXPERIMENTS

Doing PCR using primer sequences that are specific to the regions within the kanamycin cassette and the *E. coli* C29 genome could help locate the map area where the insert occurs. A Southern blot could be done using the total genomic DNA from the *E. coli* C29 strain, using the inverted repeats of the Tn903 as probes. A positive result in the Southern blot would confirm that the *E. coli* C29 genome also contains a transposon that has inverted repeats that are similar to that of Tn903, providing a potential site for homologous recombination. Also, a Southern blot could be performed on the total genomic DNA of the kanamycin resistant transformants, and using *lac*I gene probes. A positive result here would suggest that the *lac*I gene is still present within the genome, verifying that the kanamycin resistant cassette is inserting elsewhere within the *E. coli* C29 genome.

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