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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

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Specific disruption of *lacI* in *Escherichia coli* C29 cells was attempted by using the Lambda Red Recombinase plasmid pKD46. Previous attempts to disrupt *lacI* using this system may have failed due to the absence of L-arabinose in the media required for expression of the recombinase genes. In this experiment, a kanamycin PCR product with flanking regions homologous to *lacI* was used to disrupt the *lacI* gene in L-arabinose induced cells harbouring pKD46. Putative transformants were assayed for the production of β-galactosidase, in the presence or absence of the inducer IPTG, using X-gal plates and a discontinuous enzyme assay. The tested kanamycin resistant transformants were all still inducible by IPTG indicating that *lacI* was not disrupted. This suggested that the addition of L-arabinose alone did not improve efficiency of specific recombination.

Previous studies (1, 2, 6, 11) attempted to knockout the *lacI* gene from *Escherichia coli* C29 cells using the Lambda Red Recombinase system. Attempts to disrupt the *lacI* gene by inserting a kanamycin resistance gene from the pACYC177 plasmid were unsuccessful. A polymerase chain reaction (PCR) product containing the kanamycin gene was generated using primers with flanking regions homologous to *lacI* and the kanamycin gene. In all cases, the putative transformants gained kanamycin resistance but it appeared that *lacI* was still active. If *lacI* was successfully disrupted, it is expected that no difference in β-galactosidase expression levels would be observed in the presence or absence of the inducer isopropyl β-D-1-thiogalactopyranoside (IPTG). Previous studies showed a dramatic increase in β-galactosidase expression levels in the presence of IPTG despite observed kanamycin resistance, indicating the kanamycin resistance gene may have been inserted elsewhere in the genome (1).

Broekhuizen *et al.* (1) noted that previous studies, including their own, neglected to include L-arabinose in the media used for transformation of the cells harboring the pKD46 Lambda Red Recombinase plasmid. The Lambda Red Recombinase system is an expression plasmid which encodes three phage genes that directly assist in homologous recombination (*γ, β*, and *exo*) as described by Datsenko *et al*. (3). These three recombination genes are under the control of the arabinose-inducible P_{araB} promoter (also called P_{BAD}) taken from the arabinose operon. The expression plasmid also contains a fourth gene, *araC*, which is divergently transcribed under the control of the P_C promoter. This gene encodes the regulatory protein AraC. In the presence of L-arabinose, L-arabinose binds AraC so that transcription from the P_{BAD} promoter is initiated and the recombinase genes are expressed. The recombinase genes *γ, β*, and *exo* encode products Gam, Bet, and Exo respectively. Bet and Exo promote recombination by interacting with the ends of the linear DNA fragment to be inserted. Gam is an inhibitor of host RecBCD exonucleaseV activity and protects the linear DNA fragment from degradation. In the absence of L-arabinose, transcription from P_{BAD} is inhibited by the AraC response regulator.

In this experiment, we attempted to use the inducer L-arabinose to ensure expression of the Lambda Red Recombinase system genes for the disruption of *lacI*. While the transformants from previous groups showed kanamycin resistance, it was apparent that the *lacI* gene was not disrupted. Here, we hypothesized that in the presence of L-arabinose, efficiency of homologous recombination would be increased and *lacI* would be successfully disrupted with the kanamycin PCR product. However, this attempt was unsuccessful at specific disruption of *lacI* despite acquisition of kanamycin resistance.

MATERIALS AND METHODS

Plasmids. Plasmid pACYC177 is 3941 bp, containing *rep* (plasmid replication site), *kan* (kanamycin resistance cassette), and *bla* (ampicillin resistance) (9). This plasmid was isolated from *E. coli* DH5α using the Fermentas Life Sciences GeneJET™ Plasmid Miniprep Kit (#K0502). Plasmid pKD46 is 6329 bp, containing the Lambda Red Recombinase genes and *bla* (3). This plasmid was contained in *E. coil* C29 cells (1). Both plasmid strains were provided by the University of British Columbia Teaching Labs, Microbiology and Immunology department.

Bacterial strains and growth media. Both *Escherichia coli* DH5α and C29 strains were grown in Luria-Bertani broth (liquid LB: pH 7, 1% w/v NaCl, 1% w/v tryptone, 0.5% w/v yeast extract (Difco, #0127-01)) or solid media (LB solidified with 1.5% w/v agar). When required by the various protocols described below, LB was supplemented with ampicillin (amp) (100 μg/mL, Sigma, #A1593) kanamycin (kan) (50 μg/mL, Sigma, #K-4000), and 1% L-arabinose

(Aldrich, #A91 06-1009-A). C29 cells without pKD46 were used as a control in the discontinuous enzyme assay. Cultures were grown at 37°C overnight; liquid cultures were grown in a tube roller (GLAS-COL apparatus company, #RD250) at max speed.

Primers and PCR. The kanamycin cassette from the pACYC177 plasmid was amplified. The sequence of the forward primer (Lac-Kan-F) was 5'-AGGGTGGTGAATGTGAAACCAGTA ACGTTATACGATTTATGAGCCATATTCAACGG-3'. The sequence of the reverse primer (Lac-Kan-R) was 5'–TCACTG CCCGCTTTCCAGTCGGGAAACCTGTCGTGCAACTCATCGAG CATCAAATG-3'. The forward primer had 41% GC content and an approximate melting temperature of 67°C while the reverse primer had 56% GC content and an approximate melting temperature of 66°C. The forward primer, designed by Broekhuizen et al. (1), is 56 nucleotides long and contains the first 12 nt upstream from *E. coli* K12 *lacI*, the first 24 nt of *lacI,* and the first 20 nt of the kanamycin resistance cassette from the pACY177 plasmid (1). The reverse primer, also designed by Broekhuizen et al. (1), is 56 nt long and contains the last 36 nt of *E. coli* K12 *lacI*, and the last 20 nt of the kanamycin resistance cassette from the pACY177 plasmid (1). The PCR reaction was carried out using 5 µL of 10X PCR buffer, 1.25 µL (10 mM) dNTP, 2.0 µL (50 mM) $MgCl_2$, 1.0 µL each of forward and reverse primer (20 pmol/µL), 1 µL (27.5 µg/mL) plasmid pACYC177 DNA, 0.25 µL (5 U/µL) Taq polymerase, and 37.25 µL dH_20 for a total volume of 48.75 µL. The PCR reaction conditions used for 36 cycles in the Biometra T-gradient thermocycler (#1504384) were: 4 minutes at 95°C (initial denaturation), 1 minute at 95°C, 1 minute at 62.1°C, 63.9°C, 65.7°C, 67.4°C, or 70.1°C (annealing temperature gradient), 1 minute at 72°C, and 10 minutes at 72°C (final extension). A control reaction substituted water for template DNA.

Agarose gel electrophoresis. PCR products were run on a 1% agarose gel for 40 minutes at 120 V in 1X TAE buffer (8) and then stained with 0.2 µg/mL ethidium bromide for 45 minutes. Fermentas 1Kb MassRuler, GeneRuler™ (#SM0311), was loaded.

PCR product purification. The PCR product generated from annealing temperature 70.1°C was purified using Qiagen MiniElute™ Gel Extraction kit 50 (#28604).

Preparation of electrocompetent cells. The Protocol was taken from the Biorad MicroPulser™ Electroporation Apparatus Operating Instructions, section 5.1, (#165-2100). *E. coli* C29 cells harboring pKD46 were inoculated into liquid LB with amp (100 µg/mL). The protocol was modified to also include 1% L-arabinose. The culture was incubated overnight at 37°C in a tube roller. Next day, 500 mL of LB broth with amp (100 µg/mL) and 1% L-arabinose was inoculated with the overnight culture and incubated at 37°C in a tube roller until 0.6 OD_{600nm}. Tips, centrifuge bottles, microfuge tubes, and 10% glycerol were kept ice cold throughout the procedure. After centrifugation, extra care was taken to remove all residual media from the pellet. *E. coli* C29 cells were flash frozen using dry ice and placed into a pre-chilled freezer box to be stored at -80°C. Beckman model J2-21 (#258956) was used for centrifugation. The rotor was cooled to 4°C prior to centrifugation.

Transformation. Protocol was taken from the Biorad MicroPulser™ Electroporation Apparatus Operating Instructions and Applications Guide, section 5.2, (#165-2100). Two separate electroporation reactions were performed with 2 µL of both purified and non-purified PCR product. A control was prepared using 2 µL dH_2O but was not electroporated. 0.2 cm BioRad cuvettes were used on the Ec2 MicroPulser setting. The cuvettes were chilled on ice prior to electroporation. Cells and DNA were mixed and incubated on ice for 1 minute and electroporated, and then LB with 1% arabinose was quickly added. After a 1 hour recovery at 37°C, 10 µL and 100 µL of purified and non-purified PCR transformants (construct kan-arab/2007) were plated on LB and LB with kan (50 µg/mL). The non-electroporated control was also plated on LB and LB with kan (50 µg/mL). All samples were plated in duplicate and incubated at 37°C overnight.

Enzyme plate assay. To test for the expression of β-galactosidase enzyme in the presence and absence of IPTG. Transformants from the non-purified and purified PCR electroporated samples were streaked onto LB with kan (50 µg/mL) with the addition of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (40 µL at 40 mg/mL) and IPTG (40 µL at 100 mM). The same transformants were also streaked onto the same media without IPTG. The control sample was streaked onto LB with the addition of X-gal (40 µL of 40 mg/mL) with or without IPTG (40 µL of 100 mM). Plates were incubated at 37°C overnight and monitored for the production of blue pigment.

Discontinuous enzyme assay. Protocol for assaying β-galactosidase was adapted from *Manual of Experimental Microbiology for Micb 421, 2006* (8). Transformants from the X-gal plates were inoculated into 5mL of LB with kan (50 µg/mL). The controls were inoculated into 5 mL LB with amp (100 µg/mL). All cultures were incubated overnight at 37°C in a tube roller. The following day, turbidity was measured for each of the overnight cultures and appropriate dilutions were made to achieve a starting OD_{600nm} of 0.15 in fresh liquid LB media with or without IPTG (250 µg/mL). LB media was supplemented with kan (50 µg/mL) for the transformants and amp (100 µg/mL) for the controls. After three hours, cells were put on ice and the OD_{600nm} was measured for each sample to ensure approximately the same number of cells in each. Cells were removed from ice and 3 mL of culture was permeabilized with 200 µL of toluene and vortexed. After the toluene layer had separated, 400 µL of lysate was mixed with 1.2 mL of TM buffer and warmed for 5 minutes in a 30°C water bath. Then 200 µL of o-nitrophenyl-β-D-galactopyranoside (ONPG 5 mM) was added and samples were incubated at 30°C for 3 minutes. The reaction was stopped with the addition of 2 mL of 0.6 M sodium carbonate. The absorbance at 420nm was measured for each sample using the Spectronic 20D+ digital spectrophotometer (#333183).

RESULTS

Plasmid pACYC177 isolated from *E. coli* DH5α had a final concentration of 27.5 µg/mL. PCR amplification of plasmid template DNA resulted in an 880 bp band (9) as expected (Fig. 1). Of the five PCR reactions performed, samples 1, 2, 5 and 6 showed sharp and intense bands on the gel. Samples 5 and 6 fell within the optimal annealing temperature range of 67.4°C for sample 5 and 70.1°C for sample 6. Hence, the two PCR products from lanes 5 and 6 were chosen for transformation. Sample 2 (63.9°C) showed smearing and sample 3 (65.7°C) was faint and therefore these samples were not used for transformation. Following PCR, sample 5 was not purified and sample 6 was purified.

Prior to preparation of competent cells, *E. coli* C29 with pkD46 plasmid was resistant to amp and sensitive to kan. Competent cells retained the pKD46 Lambda Red Recombinase plasmid as they showed growth on LB with amp selective media. *E. coli* C29 with pkD46 plasmid that were transformed showed growth on LB, LB with kan and LB with both kan and amp. The negative control (water instead of PCR product) did not grow on LB with kan but grew on LB as expected. There was no significant difference between the total number of transformants electroporated with purified PCR product versus non-purified PCR. The average

colony count of transformed cells was 1.64×10^4 CFU/mL.

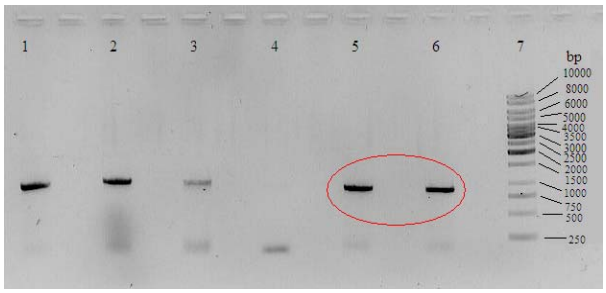

FIG. 1. 1% agarose gel electrophoresis of gradient PCR amplification products (5 μL sample + 1 μL 6× loading buffer, 6 μL of 1kb mass ladder in lane 7) of *kan* with *lacI* flanking regions from pACYC177 plasmid ran at 120V for 40 minutes. Lanes 1 to 6 had annealing temperature of 62.1, 63.9, 65.7, 63.8, 67.6, and 70.1°C, respectively. Lane 4 contains the negative control (water). The 880 bp PCR product from lanes 5 and 6 (circled) were used for transformation.

The phenotypic test on wild type *E. coli* C29 with pKD46 (control) using LB with X-gal and LB with both X-gal and IPTG resulted in blue colonies in both conditions, although the induced colonies were slightly bluer. However, white and blue colonies were expected for non-induced and induced, respectively. In addition, the putative transformants grown on LB with kan, X-gal and IPTG appeared slightly bluer than those grown on LB with kan and X-gal without inducer.

In the first trial of the discontinuous enzyme assay the negative control (wild type *E. coli* C29 harboring pKD46) did not show a significant difference in enzyme activity between induced versus non-induced samples (Fig. 2, sample 1). The transformants had higher enzyme activity in the induced sample compared to the non-induced and there was a general increase in activity for the latter transformants with IPTG. In the second trial (Fig. 3), the freshly grown wild type control C29 cells harboring pKD46 (sample 1) showed a two-fold increase in enzyme activity in the induced versus non-induced state. In addition, wild type *E. coli* C29 cells with no pKD46 (Fig. 3, sample 2) showed a three-fold increase in enzyme activity in the induced versus non-induced state. The putative transformant (Fig. 3, sample 3) showed higher enzyme activity in the induced versus non-induced state. In both trials all putative transformants showed increased levels of β-galactosidase enzyme activity when induced with IPTG (Fig. 2 and 3), where the greatest difference was a four-fold increase in expression (Fig. 3, sample 3). There was an observed increase in enzyme activity in the samples, which were read sequentially from 1 to 5, for the induced transformed samples (Fig. 2, samples 2 to 5).

DISCUSSION

The addition of L-arabinose was expected to induce expression of the Lambda Red Recombinase genes, which promote the insertion of the kanamycin resistance gene into *lacI* via site specific homologous recombination. Disruption of *lacI* would lead to constitutive expression of β-galactosidase. In addition, it is expected that the presence of the inducer IPTG should not result in increased β-galactosidase expression compared to non-induced cells.

The observation of colonies on LB with kan plates signifies that the transformed cells had acquired kanamycin resistance. This also indicates that the PCR reaction of the pACYC177 plasmid was successful in amplification of the kanamycin cassette, consistent with the presence of a single band of approximately 880 bp on the gel (Fig. 1).

The qualitative assay on the X-gal plates for the controls had unexpected results since the non-induced samples were significantly blue instead of the expected white or faint blue. Faint blue color may be seen in non-induced samples due to the leaky expression of β-galactosidase (4). Although the non-induced transformants showed constitutive expression in the absence of IPTG, as seen by the production of blue pigment, the induced samples were slightly bluer. This suggests the possibility that β-galactosidase expression is still under the control of LacI. Due to the ambiguous results for both the controls and transformants, a discontinuous β-galactosidase assay was performed.

In the first trial of the discontinuous enzyme assay, the control was consistent with the qualitative assay, where there was no significant difference between the induced and non-induced samples. Therefore, another trial was performed with freshly grown controls with and without the Lambda Red Recombinase plasmid (pKD46). Comparing trial 1 and 2, the freshly grown control cells showed more inducibility (Fig. 2, 3). In addition, the cells without plasmid showed an even greater inducibility, suggesting the plasmid might have interfered with the β-galactosidase enzyme assay. In trial 1, the increase in expression of the transformants in the induced state was possibly due to the temperature differences between the samples. Samples had progressively longer exposure to room temperature prior to incubation in the water bath. Therefore, the earlier samples had less enzyme activity due to lower temperature (8).

Transformants in both trials had higher β-galactosidase enzyme activity in the induced state. In addition, the difference between induced and non-induced was much more significant for the transformants than the controls (Fig. 2, 3). Though there was a four fold increase between the induced and

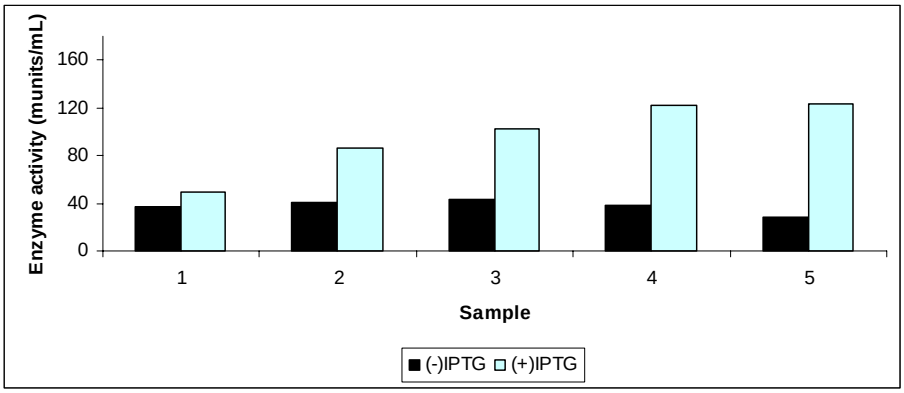

FIG. 2. Trial 1 for β-galactosidase discontinuous enzyme activity of wild type C29 cells with pKD46 (sample 1) and putative transformants (sample 2 to 5) grown in the absence and presence of IPTG.

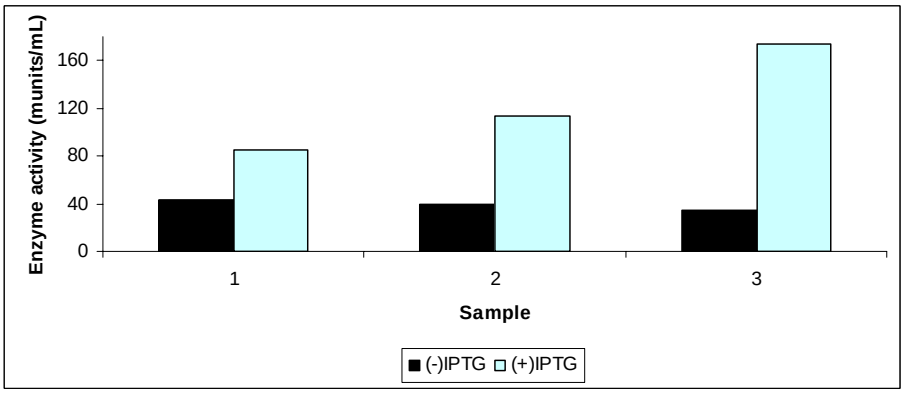

FIG. 3. Trial 2 for β-galactosidase discontinuous enzyme activity of freshly grown wild type C29 cells with pKD46 (sample 1), wild type C29 cells with no plasmid (sample 2) and putative transformant (sample 3), grown in the absence or presence of IPTG.

non-induced cells, a more significant difference was seen in previous studies (1, 2, and 6). Although the inducibility was not as great as in previous studies, the results still clearly indicate that β-galactosidase expression is regulated by the LacI repressor and hence, *lacI* was not successfully disrupted.

The presence of kan resistance indicates the kanamycin cassette was inserted elsewhere in the genome. According to Datsenko *et al*. (3) the primers should have 36-50 nt extensions that are homologous to the gene of interest for successful disruption using the Lambda Red Recombinase system. Given that the primers in this experiment had only the minimum 36 nt homology, they might not have been long enough to promote homologous recombination. In addition, the PCR product had no homology to either the Lambda Red Recombinase plasmid (pkD46) or the *E. coli* C29 genome except at the upstream and downstream regions of the *lacI* gene, as determined by bl2seq comparison available through NCBI (data not shown) (12). This indicates that the kanamycin cassette did not insert into the pKD46 plasmid or the genome via specific homologous recombination. Therefore, it is likely that there was a non-specific insertion into the *E. coli* C29 genome.

Non-specific insertion of the kanamycin gene may also have been due to low expression of the Lambda Red Recombinase genes. Although L-arabinose was included in the media, trace amounts of glucose may have been present in the undefined ingredients, tryptone and yeast extract, which would have inhibited expression from the P_{BAD} promoter (5, 10). Possible low expression of Gam, which inhibits host RecBCD exonucleaseV activity, may have resulted in increased degradation of the ends of the linear DNA fragment. Since the ends contain the homologous regions responsible for specific insertion, their truncation may have lead to decreased specific recombination. This could explain the lack of specific insertion of the kanamycin gene into *lacI*.

The results from this experiment indicate that the attempt to disrupt *lacI* using the Lambda Red Recombinase system in the presence of L-arabinose was not successful. Further adjustments to the protocol

should be made in order to achieve specific disruption of *lacI* using this system. Though this study was unsuccessful at achieving specific insertion using the Lambda Red Recombinase system, it indicates that the absence of L-arabinose was not the sole cause of difficulties in achieving recombination. The results of this study may be relevant to future endeavours using the Lambda Red Recombinase system.

FUTURE EXPERIMENTS

To increase the probability of homologous recombination, the length of the homologous region of the primers should be increased up to 50 nucleotides (3). In addition, cells should be grown in minimal media such as M9 (8) to ensure the absence of glucose, which acts as a catabolite repressor. In future experiments using the Lambda Red Recombinase system, L-arabinose should be included to ensure the expression of the recombinase genes. Furthermore, future experiments could include sequencing the kanamycin resistant transformants with primer set 1 designed by Raj (7). This primer set is designed to amplify the *lacI* gene. If the transformants have the kanamycin insert, the PCR product should be smaller than the *lacI* gene. Finally, the PCR products of both the wild type control and the transformants can be sequenced to verify the presence of the kanamycin insert.

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