Consistency and Applicability of the $\lambda$-Red Recombinase System in Escherichia coli Tested on the Target Genes lacI and phoA

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The $\lambda$-Red recombinase system has been previously used to successfully delete the lacI gene from Escherichia coli strains C29, BW2553 and MG1655, after many earlier failures. In this study, the previous successful method was tested to determine whether the system could be applied to different genes, and whether the method gives consistent results. To that end, the lacI gene in E. coli C29 was targeted to answer the second aim, and serve as a positive control, while the phoA gene was targeted to test the first aim. A kanamycin resistance cassette ($kanR$) was used as a template to create lacI-kanR constructs using the previous PCR primers, and a phoA-kanR construct using newly designed primers. Two phoA-kanR products were attempted; the second construct was not successfully created when Luk’s primers were used. The PCR products were electroporated into competent E. coli C29/pKD46 cells, which contain the pKD46 plasmid that encodes ampicillin resistance and the $\lambda$-Red recombinase. Screening for the mutants on selective media including antibiotics and colorimetric substrates showed that the target genes were still functioning. These results indicate that further work is necessary to refine the $\lambda$-Red recombinase method, as it was not able to knock out the previously successfully targeted lacI, nor the new target, phoA.

Complete understanding of the Escherichia coli pathways requires the accumulation of knowledge of not only the genome, but also what each gene product is responsible for and how it interacts with other proteins. A simple method is to simply take out, or repress, the gene and determine the resultant effects. A straightforward and permanent method is that of site-directed insertion and deletion mutagenesis (5, 7, 11). Datsenko and Wanner pioneered the phage $\lambda$-Red recombinase system, consisting of an arabinose-inducible promoter controlling the expression of the $\lambda$-Red recombinase (5). The recombinase is employed to integrate a kanamycin resistance cassette ($kanR$) into the E. coli genome, replacing a specific gene. Specificity is achieved by using PCR to add ends to the kanamycin resistance cassette that are complementary to the gene to be deleted. Thus, the target gene is deleted, replaced with a kanamycin resistance gene for easy isolation of the mutants. The replacement DNA is brought into the cell by transformation, usually electroporation. Due to the electrical pulse, many cells may die (12) but some of the surviving cells will lack the target gene and instead contain the replacement kanamycin resistance cassette.

The recombinase genes are in the plasmid pKD46, a 6329 bp plasmid that holds an ampicillin resistance gene. Controlled by the arabinose-inducible promoter, $araC$-$ParaB$, are the recombinase genes $gam$, bet and exo. With 1mM L-arabinose present in the medium, the $\lambda$-Red recombinase is expressed. pKD46 is a heat-curable plasmid, being lost at temperatures of 37°C or higher. The kanamycin resistance cassette, for PCR, is taken from pACYC177, a 3941 bp plasmid that also contains an ampicillin resistance gene (9).

LacI is a 1080 bp regulatory gene coding for a lactose repressor, which can repress the activity of lacZ, the $\beta$-galactosidase gene, whose product hydrolyzes $\beta$-galactosides into monosaccharides. With lacI replaced by a kanamycin resistance gene, the expression of lacZ should greatly increase compared to wild type. Previous groups (1, 4, 6) attempted to delete lacI, and failed due to a lack of arabinose needed to induce the pKD46 plasmid to produce the recombinase, and growing the strains at 37°C, which caused a loss of pKD46 through heat curing. These errors avoided, another group targeted a different gene, phoA with the $\lambda$-Red recombinase system, to no avail (9).

PhoA is an alkaline phosphatase enzyme, which aids the bacterium by creating a source of free phosphate. With phoA replaced by $kanR$, the E. coli would not be able to create free phosphates, limiting its growth to the free phosphate present in the medium. An even later group, Beamish et al, finally succeeded in deleting the lacI gene of three E. coli strains; BW2553, MG1655 and C29 (2).

It was hypothesized that with Beamish et al’s success,
their final methodology would be workable for different *E. coli* genes. To test that, and to ensure Beamish et al.’s success was not a one-time fluke, *phoA* was targeted in this study, using their method with some small differences. *lacI* was also targeted, to serve as a positive control.

**MATERIALS AND METHODS**

*Strains.* *E. coli* strain DH5α/pACYC177 was used to obtain the plasmid, pACYC177, containing a kanamycin resistance cassette. *E. coli* strain MG1655/pKD46 was used to obtain the plasmid, pKD46, which contains the λ-Red recombine system genes. The strain properties can be seen in ref. 2. *E. coli* strain C29 was used to create a C29/pKD46 strain, by electroporation of the pKD46 plasmid into C29 competent cells. All strains were from the UBC MICB 421 Teaching Lab stock collection, Department of Immunology and Microbiology, University of British Columbia. All growth was at 30°C, except to test C29/pKD46 at 42°C, where the ampicillin resistance is lost by heat curing of the plasmid.

*Plasmids.* *E. coli* strain MG1655/pKD46 and DH5α/pACYC177 were grown overnight in LB broth to a turbidity between 1 and 2 OD600. The PureLink™Quick Plasmid Miniprep kit (Invitrogen, Carlsbad, CA, K2100-10, lot# 4908) was used to isolate and purify the plasmids pKD46 and pACYC177. pACYC177 was restriction digested with BglI enzyme (New England BioLabs, R0143S) and 1X NEBuffer 3 at 15°C for 1 hour, and the reaction stopped with 20 minutes at 65°C. The digested pACYC177 solution was diluted 1:60 in sterile distilled water. Plasmid concentrations were measured in an Ultrospec 3000 (Biochrom).

*Media.* All strains were grown in Luria-Bertani broth (LB), composed of 1% Bacto tryptone, 1% NaCl (EMD, SX0420-1), 0.5% w/v yeast extract (BD Bacto, Sparks, MD, 212750) with 1 mM L-arabinose (Sigma Aldrich, A91906-100G, lot# 12925LA). 1.5% agarose was added to make LB Agar. Select Agar (Invitrogen, Carlsbad, CA, K2100-10, lot# 4908) was used to isolate and purify the plasmids pKD46 and pACYC177. pACYC177 was restriction digested with BglI enzyme (New England BioLabs, R0143S) and 1X NEBuffer 3 at 37°C for 1 hour, and the reaction stopped with 20 minutes at 65°C. The digested pACYC177 solution was diluted 1:60 in sterile distilled water. Plasmid concentrations were measured in an Ultrospec 3000 (Biochrom).

*Selection media.* For the *E. coli* strain MG1655/pKD46, C29/pKD46, DH5α/pACYC177 and mutated C29/pKD46 cells, ampicillin at 100 μg/ml was added. For the latter two, kanamycin was also added, at 50 μg/ml. *phoA* mutants were identified by adding 5-bromo-4-chloro-3-indoyl-phosphate, 47.5 μg/ml, (BCIP, Sigma-Aldrich, St. Louis, MO, B6149-50MG, lot# 014K1022) to the media. 5-bromo-4-chloro-3-indoyl galactopyranoside (X-gal; Sigma Chemical Company, St. Louis, B-4252, lot # 82F-0099) was used at 40 μg/ml, to identify *lacI* deleted-C29/pKD46 cells.

**Primers.** Primers to create a *lacI-kanR* product were used from a previous group (2). Primers for a *phoA-kanR* product were used from a previous group (11), though they had not been successful in transformation, therefore new primers were also designed for a *phoA-kanR* product (Table 1), and tested with M-fold analysis (8) and the National Center for Biotechnology Information (NCBI) b2seq program at http://blast.ncbi.nlm.nih.gov/Blast.cgi The new primers were constructed by Integrated DNA Technologies (San Diego). The primers used to make the *lacI-kanR* and *phoA-kanR* products were also used in confirmation PCR.

**PCR to create kanR-target product.** The reaction volumes of 25 μL were made up in 0.2 mL domed PCR tubes (BioRad laboratories, Hercules CA, TW10201), containing 1X PCR buffer; 0.2 mM dNTP; 1 mM MgCl2; 1 unit Taq polymerase; 0.5 μg of the digested pACYC177 containing the kanR gene and sterile distilled water. Standard PCR supplies were from Invitrogen. Negative controls included all reagents and primers, but no template DNA. Positive controls included all reagents, but used primers and template DNA from Invitrogen: the Topocloning Control PCR Primers (46-0100) and Control DNA (46-0118). The primer concentrations and PCR conditions are in Table 2. All PCR runs started off with 95°C for 10 min; then 35 cycles of: 95°C for 1 min, annealing (see Table 2 for variations), 72°C for 1.5 min; and the last extension at 72°C for 10 min. Reactions were run in a Biometra T-gradient Thermoblock or a BioRad GeneCycler.

6 μL of each PCR mixture was run on a 1.5% Amresco 3:1 agarose gel (Invitrogen, Carlsbad, CA, 15510-027, lot# 137923) in 1X TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.5), in a Horizon® 58 Gel box (Life Technologies, Gibco, BRL). The gels were stained in 0.5% ethidium bromide for at least 25 minutes, and then visualized in a Multiiimageight Alphalmager™ Cabinet (Alpha Innotech Corporation).

**Competent cells.** *E. coli* C29 and C29/pKD46 cells were made competent in a method similar to that described by Beamish et al (2). 5 mL of LB broth was inoculated and grown overnight at 30°C (with 100 μg/ml ampicillin for the C29/pKD46 cells). The next day, 100 μL of LB broth with ampicillin if applicable) was inoculated with 5 μL of the overnight culture, and grown at 180 rpm, 30°C. Once OD600 = 0.4 was reached, the cells were chilled on ice for 15 minutes,

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Description</th>
<th>Nucleotide Sequence (5' to 3')*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>lacI-kanR</em> forward, for PCR product, from ref. 2</td>
<td>TACGCCCCGAAGAGAGCTCAATTCAAGGTTGGTGAATGTGagcataatctacgagggg</td>
</tr>
<tr>
<td>2</td>
<td><em>kanR-lacI</em> reverse, for PCR product, from ref. 2</td>
<td>GCCTAATGATGGATGCTAACCACATTGATGCCTGCGCCtggaaaaactcatcgagac</td>
</tr>
<tr>
<td>PhoA-KanR f</td>
<td><em>phoA-kanR</em> forward, for PCR product</td>
<td>ACAAAGCATTATTTGCAgacctatctatgcttgctagcc</td>
</tr>
<tr>
<td>PhoA-KanR r</td>
<td><em>kanR-phoA</em> reverse, for PCR product</td>
<td>CAGAGCGGCTTTCATGGTGTAaaactcatcgagcatc</td>
</tr>
<tr>
<td>Apsc-KanR F</td>
<td><em>phoA-kanR</em> forward, for PCR product, from ref. 9</td>
<td>CACGGGCGAGACTTAcatctatgaacaaata</td>
</tr>
<tr>
<td>Kan-R-Apsc R</td>
<td><em>kanR-phoA</em> reverse, for PCR product, from ref. 9</td>
<td>ATAGCACCATCCCtcaacatttaacaaac</td>
</tr>
</tbody>
</table>

*Upper case letters refer to *lacI* overlap; lowercase letters refer to *kanR*; uppercase bold letters refer to *phoA*.

**TABLE 1.** Primers sequences.
TABLE 2. PCR conditions for making kanR-target

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Primer concentration (μM)</th>
<th>Annealing temperature (°C)</th>
<th>Annealing time (sec)</th>
</tr>
</thead>
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<tr>
<td>lacI</td>
<td>0.02</td>
<td>52</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>52</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>0.40</td>
<td>52</td>
<td>45</td>
</tr>
<tr>
<td>PhoA (PhoA-KanR)</td>
<td>0.80</td>
<td>52</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>0.80</td>
<td>56</td>
<td>60</td>
</tr>
<tr>
<td>PhoA (Apase-KanR)</td>
<td>0.02</td>
<td>56</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>0.06</td>
<td>56</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>52</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>56</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>56</td>
<td>60</td>
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*Each row contains a different variation. For example, for the LacI primers, the 0.4 μM primers were used at an annealing temperature of 52 °C for 45 seconds, and in another trial, at 40 °C for 1 minute.

†The middle PhoA row refers to the newly designed primers; the last PhoA row refers to Luk’s primers, ref. 9.

then centrifuged at 4000 rpm in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (DuPont Instruments) with a Sorvall SLA-1500 SuperLite rotor, for 10 minutes at 4°C. The pellet was re-suspended in 40 mL ice-cold 10% glycerol (BDH, B28454-76, lot# 38152849), then centrifuged again at 4°C for 10 minutes, in a Sorvall SS-34 rotor. After re-suspension in 5 mL 10% glycerol, it was centrifuged in the Sorvall SS-34 rotor, at 4°C for 10 minutes, and re-suspended in 1 mL of 10% glycerol. The competent cells were stored in 50 μL aliquots at ~80°C.

**Electroporation.** An aliquot of C29 competent cells was thawed on ice, while a 0.2 cm electroporation cuvette (BioRad, CA) was pre-chilled on ice. 40 μL of cells and 2 μL of pKD46 DNA were mixed in a microfuge tube and incubated on ice for 1 minute, and then transferred to the electroporation cuvette. A MicroPulser (BioRad, CA, 165-2100) was used to shock the mixture with 2.5 kV, in under 5 msec. Immediately, 1 mL of LB broth (no antibiotics) was added, and the mixture was incubated at 30°C for at least 2 hours, and up to 3 days.

**TABLE 3. C29/pKD46 and DNA volume variations for electroporation.**

<table>
<thead>
<tr>
<th></th>
<th>C29/pKD46 (μL)</th>
<th>DNA (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>20</td>
</tr>
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<td></td>
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<tr>
<td></td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
</tr>
</tbody>
</table>

Similarly, *E. coli* C29/pKD46 cells were thawed and mixed with the PCR-made products. 2 μL of the lacI-kanR DNA was used to electroporate 40 μL C29/pKD46 cells. Different combinations with the phoA-kanR DNA were tried (Table 3). To test for competency, an aliquot of competent *E. coli* C29/pKD46 cells were also electroporated with the pACYC177 plasmid, and plated on media with kanamycin (50 μg/ml) and ampicillin (100 μg/ml) 2 hours – 4 days later.

**Selection.** The *E. coli* C29 cells transformed with pKD46 were plated on LB agar with 100 μg/ml ampicillin and grown at 30°C for several days. A pure colony was isolated to make *E. coli* C29/pKD46 stock. The *E. coli* C29/pKD46 cells transformed with the phoA-kanR or lacI-kanR DNA were plated (400 μL) on LB agar with kanamycin (50 μg/ml) and ampicillin (100 μg/ml). The C29/pKD46 prospective lacI mutants were grown with 40 μg/ml X-gal added, the phoA putative mutants with 47.5 μg/ml BCIP. Colourless colonies were selected for and re-plated.

**RESULTS**

**Plasmid isolations.** The pKD46 plasmid was successfully isolated from the *E. coli* strain MG1655/pKD46 (data not shown). This plasmid was properly incorporated into the *E. coli* strain C29, resulting in strain C29/pKD46, which has ampicillin resistance. When plated on an LB plate with ampicillin and BCIP, the cells grew and became blue, proving they were phoA constitutive (data not shown). When the cells were incubated at 42°C, there was no growth seen due to heat loss of the plasmid, proving the ampicillin resistance was due to the plasmid, and not a mutation (data not shown). This strain, C29/pKD46, was then made competent. To ensure the collected cells were viable and competent, some *E. coli* C29/pKD46 were then transformed with pACYC177, as a positive control against further transformations. Those transformed cells, when plated on media with ampicillin and kanamycin, grew, ensuring the cells were competent (data not shown). pACYC177 was transformed cells, when plated on media with ampicillin and kanamycin, grew, ensuring the cells were competent (data not shown). pACYC177 was transformed with kanamycin (50 μg/ml) and ampicillin (100 μg/ml) 2 hours – 4 days later.

**PCR X-kanR products.** Figure 1 shows the lacI-kanR product of 891 bp. The product in Lane 5 was cleaner, so that was used in the ensuing transformations. This product was obtained by 0.4 μM primers, in a program of 5 minutes at 95°C; 35 cycles of 1 minute at 95°C, 1 minute at 40°C, and 90 seconds at 72°C, followed by a final extension of 10 minutes at 72°C.

In all the PCR trials completed using Luk’s Apase-KanR F and KanR-Apase R primers (9), there was no phoA-kanR PCR product seen, though the positive control worked in all cases. Figure 2A contains an example electrophoresis gel with PCR results obtained using Luk’s primers.

**A phoA-kanR product was obtained using the newly designed PhoA-KanR f and PhoA-KanR r primers.** The product was the correct expected size of 851bp (Fig. 2B). The products run in lanes 6, 8 and 10 (annealing temperatures 37.5°C, 44°C and 51°C) were used in...
1 2 3 4 5

FIG. 1. lacI-kanR PCR product. Lane 1 contains the λ/HindIII ladder; Lane 2 a negative control (with leakage from lane 3); Lane 3 the positive control; Lanes 4 & 5 the LacI-kanR product.

subsequent transformations, with transformation variations as seen in Table 3.

**PCR product transformations.** No successful transformation of *E. coli* C29 with neither the lacI-kanR nor the phoA-kanR PCR product was observed. The transformations were plated on selection media, LB with ampicillin and kanamycin, and either BCIP or X-gal. Transformation samples outgrown in broths that were plated within 5 hours of the transformation showed no colonies (data not shown).

The lacI-kanR product was transformed with 2 μL into 40 μL of competent *E. coli* C29/pKD46 cells in LB broth, at 2.52 kV for 2.70 msec and plated three days later. There was no growth by 24 hours later. When it was plated two days later (5 days post transformation), after 24 hours incubation, there was growth. Both white and pale blue colonies were seen (data not shown). Isolated white colonies, re-streaked, resulted in a mixture of white and pale blue (Fig. 3). When 10 μL of a phoA-kanR PCR product was transformed into 40 μL of competent *E. coli* C29/pKD46 cells, at 2.49 kV for 3.80 msec, and plated three days later, a mix of white and deep blue colonies was seen (data not shown). The whitest colonies were isolated and re-streaked, and after time for growth, examined. Some colonies did not grow; most colonies that grew were not pure white (Fig. 4). The whitest most isolated colonies were chosen for PCR.

**Confirmation PCR.** When pure white colonies were selected for confirmation PCR with the primers that were used to make the kanR-X products used for transformation, the bands from the three putative phoA mutants and the three putative lacI mutants were all identical to the control, which used parental *E. coli* C29, indicating that these were not mutants (Fig. 5).

Also, possibly the phoA-kanR bands leaked over into the putative lacI mutants' lanes: the lacI-kanR product bands were at the same height as the phoA-kanR product bands, and faded out from right to left.

**DISCUSSION**

The hypothesis, that Beamish *et al*.'s method (2) leads to uniform results and is applicable to genes other than LacI, was not supported by this study. There are two main stages to the λ-Red recombinase method to mutate or delete genes. The first stage, creating via PCR a gene product that can be melded with the target genome to replace the original gene, was successful. A lacI-kanR product was made using Beamish *et al*.'s primers (2); a phoA-kanR product was made using newly designed primers. The PCR product sizes were originally calculated by manually counting the nucleotides which would be included in the PCR product. When the samples were run on an agarose gel, the correct sizes of 891 bp and 851 bp, respectively, were seen.

What is interesting to note is the PCR method for the two primer sets that had been employed in previous years to create these products differed from what had been successful previously. The lacI-kanR product was previously obtained by 0.4 μM primer and a PCR run of 5 minutes at 95°C; 35 cycles of 45 seconds at 94°C, 45 seconds at 52°C, and 90 seconds at 72°C; followed by a final extension of 10 minutes at 72°C (2). When that was tried, however, no product resulted. Product was obtained when 1 minute was used instead of the 45 second times; 95°C instead of 94°C; and 40°C instead of 52°C. Though the same concentrations and brands of reagents were used, it is possible there were differences in quality or purity between lots, leading to these different results. However, a lacI-kanR product of correct size was finally obtained.

What was more problematic was that a phoA-kanR product using Luk’s primers and method was not made. None of the different variations tried (Table 2) resulted in a visible product, when the sample was run on an agarose gel and viewed. Thus, Luk’s work on attempting to delete phoA could not be continued with his primers. The newly designed primers for a phoA kanR product did, after the variations in Table 2, result in a product of the correct size (Fig. 2B).
The second stage to the λ-Red recombinase method involves transforming electrocompetent cells that contain the recombinase gene, with the PCR product to be integrated. There was no success here, with neither the lacI-kanR nor the phoA-kanR products. When some of the competent E. coli C29/pKD46 cells, containing ampicillin resistance, were transformed with pACYC177 (kanamycin and ampicillin resistance) and grown on LB media with ampicillin and kanamycin, cells did grow (Fig. 1B), indicating the cells were both viable and able to be transformed. Therefore, the problem does not reside with the cells, nor with the selective conditions.

The problem could, ostensibly, lie with the PCR products. The primers were designed off of the genome of E. coli MG1655, as the C29 strain is not available. The differences between the genomes, for the two genes of lacI and phoA, should not be so great, however, as to hinder recombination. Other proof that the primers should be sufficient includes three facts. Since only one band for each product was seen, there is little chance that the primers matched with a second area on the genome, and formed a product of the expected size. As well, in the design of PhoA-KanR f and PhoA-KanR r, the primers were blasted against the E. coli MG1655 genome, using the NCBI’s bl2seq program; no other complementary areas were found. Thirdly, the lacI-kanR primers (primers #1, #2) were used for successful lacI mutation previously (2), so there can be no problem with them. Thus, the PCR product was probably not at fault.

Therefore, the problem might reside with the transformation protocol. Transforming in small pieces of linear DNA that must integrate into the genome is 10^3 to 10^4 less efficient a process, compared to electroporating in an independent plasmid (3). Accordingly, in the 1 mL of transformed broth, there may have been a very few cells that were successfully transformed, but not plated. This is not a great possibility, as up to 400 μL of each broth were plated, and even if only a few cells had transformed, the colony formed would be visible. It may be necessary to not focus on plating greater quantities, but instead make the electroporation more efficient, by varying: competent cells’ volume, DNA volume, and perhaps moving away from the suggested program of 2.5 kV. The transformation efficiency depends on other factors as well that could be looked at, such as the temperature, resistance and the treatment of the DNA sample (10).

If not the transformation, the recombination procedure might be at fault. Even if the cells did successfully take up the DNA, it must be integrated into
the host DNA by use of the recombinase encoded by pKD46. The positive control test of transforming in pACYC177 into the E. coli C29/pKD46 cells only shows that the cells were competent; the pACYC177 plasmid did not need to be integrated to be expressed. Therefore, a positive control for recombination would be necessary to test if the recombinase is not working properly, or if the cell is destroying the foreign DNA. A positive control consisting of DNA that has previously been successfully integrated should be used.

Another possible explanation for the lack of transformed cells may be that the cells take a long time to incorporate and express the changes. At first, the broths were plated 1-2 hours after transformation, as suggested by the previous groups (2, 9) and the BioRad MicroPulser handbook (3). Those plates showed no growth, however. When the broths were plated 3-5 days or longer after transformation, there were some colonies visible. Perhaps waiting even longer before plating would yield greater numbers of transformed cells. Another point was that the blue colour, due to BCIP or X-gal hydrolysis, took longer to develop. Some colour was noticed to have developed after placing the plate at 4°C for several days, after having been incubated at 30°C for growth.

A human mistake noted too late for correction was that white colonies were picked as lacI mutants, and used in the confirmation PCR. Since lacI is a repressor, a mutant should have increased β-galactosidase activity, and instead any intense blue colonies should have been isolated as putative lacI mutants. Thus, it is understandable that the confirmation PCR for lacI mutants had the same results as for the parental E. coli C29 control, as they certainly retained the lacI gene. Though this opens the possibility that a mutant did arise, but was not isolated, this is likely not the case. On the selection plates for the lacI mutants, there were only white and pale blue colonies seen, none with a deep blue colour which is indicative of a lacI mutation.

In conclusion, the hypothesis that the λ-Red recombinase system can be confidently used to mutate specific genes was not supported. However, the positive control, the lacI mutation successfully performed by a previous group, did not work properly either. This could indicate that the λ-Red recombinase method is so fastidious to be unreliable, or that there is something about the transformation and selection processes that needs to be further refined.

**FUTURE EXPERIMENTS**

The next step is to continue work on supporting or disproving this hypothesis. More transformations, at the same time, with variations in DNA and competent cell
volume should be done, to determine whether the \(\lambda\)-Red recombinase method truly failed, or whether the very low transformation efficiency is the reason for the negative results. It is also important to include some form of a positive control for recombination, to assess that the cells can not only take up foreign DNA but also integrate it.

It is very important to be well organized and efficient with regards to time, so the PCRs to create the DNA product to be electroporated into the cells should be started early, to allow time for re-optimization, as was necessary in this study. Once cells are transformed, they should be grown in broth with no antibiotics for at least 4 days before plating, then at least two days should be allowed for growth, with a further two days at 4°C to allow for blue colour development.

The groups who continue on this work should not design new primers, as that would leave unanswered questions. Instead, the transformation and selection process should be focused on making these primers work, with accompanying positive and negative controls used.

Once a mutant is isolated, the confirmation PCR should be done, as well as enzyme activity assays to determine that the target enzyme is completely knocked out.

ACKNOWLEDGEMENTS

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REFERENCES