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Analyzing the efficacy of the λ-red recombinase system by site-directed mutagenesis of *pho*A and *lac*I in *Escherichia coli*

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Jaeger *et al.* originally conducted an experiment to deactivate the lactose repressor gene (*lac*I) in *Escherichia coli* C29 using the λ-red recombinase system following the success of Datsenko *et al.* with the same system. The results obtained by Jaeger *et al.* showed that while integration of the kanamycin-resistance cassette occurred, it did not lead to deactivation of *lac*I, suggesting that the system had mistargeted the integration of the cassette. Many of the follow-up studies carried out were not able to account for the lack of success in deactivating the *lac*I gene. This project extended the previous studies by looking at the effectiveness of the λ-red recombinase system in two variations of the original experiment performed by Jaeger *et al.* In one experiment, the λ-red recombinase system was used to disable the alkaline phosphatase gene (*pho*A) in *Escherichia coli* C29 to demonstrate that mistargeting was not gene specific. In the second experiment, the same system was used to deactivate *lac*I in *Escherichia coli* MG1655 to determine if the system was strain-specific. Similar to the previous studies, the kanamycin-resistance cassette from pACYC177 was amplified and used for site-directed disruption of both *lac*I and *pho*A. The transformation steps carried out in this study were successful, yielding kanamycin-resistant colonies. A brief screening test performed on C29 immediately showed that the *pho*A gene was still functional and not disrupted. A similar screening test performed on MG1655 initially suggested that *lac*I was disrupted, suggesting constitutive β-galactosidase production. However, a β-galactosidase assay showed that the use of IPTG resulted in elevated levels of β-galactosidase production. These results indicate that the λ-red recombinase is mistargeting the kanamycin-resistance cassette since disruption of the targeted genes was not achieved.

β-galactosidase is a metalloenzyme responsible for the hydrolysis of lactose into glucose and galactose (11). Production of the protein is mediated by the well-studied lac operon consisting of lacZ, lacY, and lacA. The lacZ gene encodes for β-galactosidase while lacY and lacA encode for permease and transacetylase respectively (1). Regulation of this operon is carried out by the *lac*I gene product which in its active state will bind upstream of the lac operon. This action blocks the RNA polymerase from synthesizing the mRNA for β-galactosidase.

Alkaline phosphatase is another well studied metalloenzyme which is responsible for non-specific hydrolysis of a number of phosphomonoesters (7). In bacteria such as *E. coli*, expression of the enzyme is induced when the microorganism faces phosphate starvation (5). Regulation of the phosphate regulon is carried out by a 2-component phosphorelay system via PhoB and PhoR. In this system, the PhoR sensor modifies the PhoB activator to activate transcription of the phosphate regulon (13).

Many methods have been used in the past to study both of these systems. A more recent method which has been employed for studies in molecular biology is the λ-red recombinase system. The λ-red recombinase system is a simple and useful method that is often used for site-specific inactivation of genes (2). This system consists of the genes γ, β, and exo. The function of the γ protein is to inhibit RecBCD activity, allowing for the presence of linear DNA fragments (12). The exo protein is a dsDNA-dependent exonuclease that degrades the DNA from 5' to 3' to leave 3' overhangs (14). Beta binds to the resulting ends to form an intermediate for the recombination process (14).

In a previous study, Jaeger *et al*. (6) attempted to use the λ-Red recombination system to deactivate the *lac*I gene in order to cause constitutive β-galactosidase production in a C29 strain of *E. coli*. Deactivation of the *lac*I gene would eliminate the requirement for isopropyl-β-D-1-thiogalactosidase (IPTG) for inducing β-galactosidase production. As part of their experiment Jaeger *et al*. intended to insert a kanamycin-resistance cassette directly into the *lac*I gene (6). While integration of the kanamycin-resistance cassette was successful, as demonstrated by the growth of kanamycin-resistance C29, complete deactivation of the *lac*I gene was not observed. Instead, Jaeger *et al*. (6) saw that upon IPTG induction, the test strains produced β-galactosidase at levels much higher than basal levels. Follow-up studies were conducted in which the initial

experiment was repeated, but the specific amino acids targeted for mutation were slightly altered (11, 3). However, the results from those experiments were similar to those from Jaeger *et al.* (6) in which *lac*I was not deactivated. In another follow-up study, Raj (8) sequenced the chromosomal DNA of the modified *E. coli* C29 cells obtained from Jaeger *et al*. (6) and found that recombination did not take place at the site originally specified.

This study continued on from previous experiments by looking at the efficacy of the λ-Red recombination system. Two variations of the experiment originally done by Jaeger *et al*. were performed simultaneously. The first experiment focused on using the recombination system to disrupt the *lac*I gene in the strain MG1655 in order to determine if the system was specifically faulty towards the C29 genotype. This experiment also addressed a previous hypothesis regarding the experiment originally executed by Jaeger et al. (6) in that a second *lac*I site may have been present in the C29 strain that was being used. Targeting of the kanamycin-resistance cassette was suggested to have only occurred in one of the two *lac*I sites, thus failing to stop *lac*I expression. The MG1655 strain used for this experiment, however, is known to contain only one *lac*I site. Performing the experiment on the MG1655 strain will provide insight as to whether a second *lac*I site was responsible for the results previously observed. The second experiment focused on disrupting the alkaline phosphatase (*pho*A) gene using the same system to demonstrate that mistargeting was not specific for *lac*I.

The plasmid used for supplying the λ-red recombinase system for site-directed mutagenesis of both C29 and MG1655 was the 6329bp pKD46 (Fig. 1).

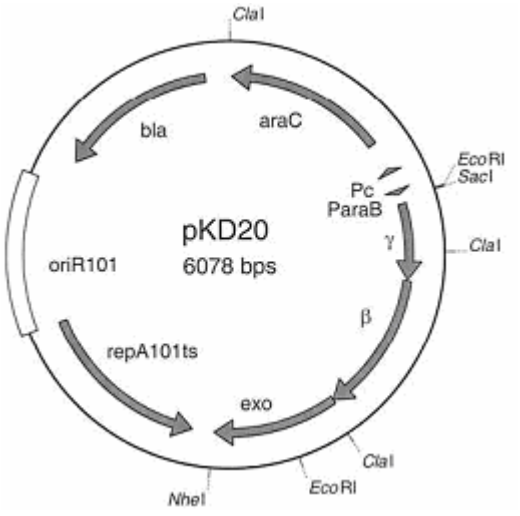

FIG. 1 Restriction map of pKD20. pKD20 is similar to pKD46 except for the exclusion of a λ tL3 terminator which is present in pKD46 (6).

This self-sustainable plasmid also contained an ampicillin-resistance gene for selection purposes. The plasmid which supplied the kanamycin-resistance cassette was the 3941bp pACYC177 (Fig. 2).

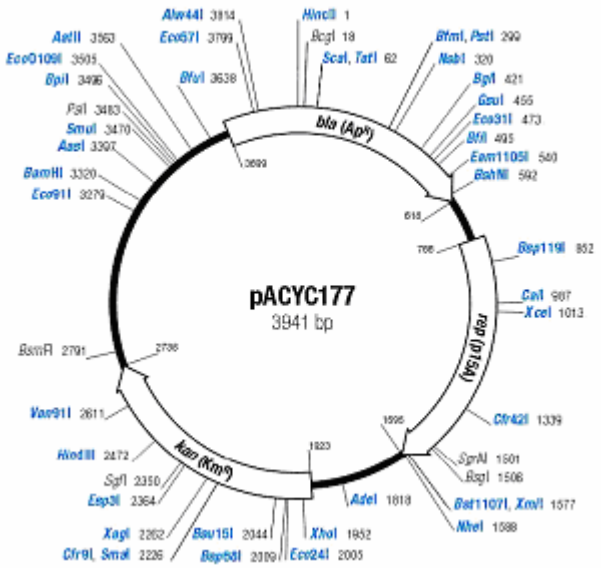

FIG. 2 Restriction map of pACYC177 plasmid with the kanamycin-resistance gene cassette (6).

This experiment was designed to allow for disruption of both *lac*I and *pho*A using the λ-red recombinase system. However, the main purpose of the project was to systematically eliminate potential explanations as to why the system did not work for previous studies. The hypothesis being tested was that the λ-red recombinase system is not strain or gene-specific, and that another factor must have been responsible for the lack of success in disruption of the targeted genes.

MATERIALS AND METHODS

Plasmids. pKD46 was isolated and purified from the *E. coli* strain BW25113 using the Qiagen Qiaprep Spin Miniprep kit (Qiagen Inc., Cat. 27106). pACYC177 was isolated and purified from *E. coli* DH5alpha using the Qiagen Qiaprep Spin Miniprep kit (Qiagen Inc., Cat. 27106).

Media. LB broth (1%w/v tryptone, 0.5%w/v yeast extract, and 1%w/v NaCl) was used for growing up *E. coli* C29 and *E. coli* MG1655 and their variants throughout the entire study.

Strains. Modified *E. coli* C29 cells containing the plasmid pKD46 (UBC Teaching Lab frozen stock, Department of Microbiology and Immunology, University of British Columbia) were grown overnight at 37ºC with shaking in LB broth containing 50ug/ml ampicillin. Following transformation with the

kanamycin-resistance cassette from pACYC177, isolates were grown overnight at 37ºC on solid LB agar with 50ug/ml kanamycin. When testing for the presence of alkaline phosphatase, cultures were grown on LB kanamycin plates at a final concentration of 50ug/ml with 2mg of 5-bromo-4-chloro-3-indolyl phosphate.

Wild type MG1655 (UBC Teaching Lab frozen stock, Department of Microbiology and Immunology, University of British Columbia) was initially grown in LB broth and incubated overnight at 37ºC with shaking. MG1655 cells transformed with the pKD46 plasmid were streaked on LB agar plates with ampicillin at a final concentration of 100ug/ml and grown overnight at 37ºC. After MG1655 cells containing pKD46 plasmids were transformed with pACYC177 plasmids, the cells were grown overnight at 37ºC on LB agar plates with a final kanamycin concentration of 50ug/ml and 1.6mg of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside.

Primers and PCR. Two sets of primers were designed and constructed – one for testing *lac*I deactivation and the other for testing alkaline phosphatase deactivation. Each of the primers was constructed as 30-nucleotide hybrid primers. The primers were designed such that the outer 15 nucleotides targeted either the *lac*I or the *pho*A gene while the inner 15 nucleotides targeted the kanamycin-resistance cassette from pACYC177 plasmid.

The sequence of the forward primer for studying *lac*I deactivation, LacI-KanR F, was 5'-TCCTGTGTGAAATTGGGTTGATGAGAGCTT-3'. The sequence of the reverse primer, KanR-LacI R, was 5'-CGGAAGAGAGTCAATATGAGAGCTTTGTTG-3'. The forward primer was designed to contain 15 nucleotides that were 115 nucleotides upstream of the *lac*I gene, followed by another 15 nucleotides specific for a region 241 nucleotides upstream of the kanamycin-resistance cassette. The outer 15 nucleotides of the reverse primer were specific for a region 69 nucleotides downstream of the *lac*I gene, while the inner 15 nucleotides were specific to an area 182 nucleotides downstream of the kanamycin-resistance cassette.

For the study of alkaline phosphatase deactivation, the forward primer Apase-KanR F had the sequence 5'-CACGGCCGAGACTTATCATCATGAACAATA-3'. The reverse primer KanR-Apase R had the sequence 5'-ATAGCACCATCCCTCCAACCAATTAACCAA-3'. The forward primer was made to first include 15 nucleotides at 65 nucleotides upstream of the *pho*A gene. The remaining 15 nucleotides of the forward primer targeted an area 53 nucleotides upstream of the kanamycin-resistance cassette. Similar to the reverse primer for *lac*I deactivation, the 5' end of the reverse primer targets an area 105 nucleotides downstream of the *pho*A gene, while the 3' end targets an area 10 nucleotides downstream of the kanamycin-resistance cassette.

Each of the 15 segments specific to either the *lac*I or *pho*A gene was blasted (NCBI, BLAST) against the *E. coli* K12 total genome to ensure specificity. The segments corresponding to the kanamycin-resistance cassette were also blasted against the total pACYC177 plasmid sequence to ensure specificity. Both sets of primers were sent to the Nucleic Acid Protein Synthesis Unit (NAPS, UBC) for synthesis. The lyophilized primers that were received were resuspended in sterile distilled water. The concentration of the resuspended primers was determined by spectroscopy at A620.

The PCR mix used for amplification consisted of 1X PCR buffer, 0.2mM dNTP, 1.5mM $MgCl_2$, 1µM forward and reverse primers, 1.25units Taq polymerase (Invitrogen Life Technologies, Cat. 18038), and 1ng isolated pACYC177 DNA, to a final working volume of 50µl.

Optimal amplification of the kanamycin cassette for both studies was determined using a temperature-gradient thermocycler. The PCR program used was 1 cycle of initial denaturation at 95ºC for 4 minutes, 40 cycles consisting of denaturing at 95ºC for 1 minute, annealing at a range of temperatures for 1 minute, extension at 72ºC for 1 minute, followed by 1 cycle of final extension at 72ºC for 10 minutes. The annealing temperatures tested were 40, 41.9, 46.4, 52.1, 55.9, and 59.5ºC.

Preparation of Electrocompetent Cells. Both C29 and MG1655 strains were grown overnight at 37ºC as 5ml seed cultures in LB broth. The seed culture containing the C29 strain already carrying pKD46 also contained 50ug/ml ampicillin. Overnight cultures were then inoculated into 250ml of LB broth at 37ºC with shaking. Cultures were chilled on ice for 20 minutes when cells had reached an OD_{600} between 0.5 and 0.7. Cells were spun down in pre-chilled centrifuge tubes at 4000 xg for 15 minutes at 4ºC. The remaining pellets were resuspended in 250ml of ice-cold 10% glycerol. The suspensions were centrifuged at 4000 xg for 15 minutes at 4ºC. The remaining pellets were resuspended in 125ml of ice-cold 10% glycerol. The suspension was re-centrifuged at 4000 xg for 15 minutes at 4ºC. The pellets were resuspended in 10ml of ice cold 10% glycerol and transferred to a 30ml sterile oakridge tube. The tubes were centrifuged at 4000 xg for 15 minutes before resuspending the pellet in a final 1ml volume of ice cold 10% glycerol in 100µl aliquots. Aliquots were flash-frozen using dry ice and stored at -80ºC.

Electroporation. Forty microlitres of wild-type electrocompetent MG1655 cells were mixed with 2µl of purified pKD46 in pre-chilled 1.5ml microfuge tubes and incubated on ice for 1 minute. The resulting mixtures were transferred to pre-chilled BioRad 0.2cm

electroporation cuvettes (Bio-Rad Laboratories, Inc., Cat. 165-2082). The cuvettes were placed in the BioRad MicroPulser (Bio-Rad Laboratories, Inc., Cat. 165-2100) and pulsed at 2.5kV for 1 second according to the manufacturer's manual. Cells were immediately resuspended in 1ml of LB pre-heated to 37ºC. Cell suspensions were grown at 37ºC with aeration for 1 hour. One hundred microlitres of each mixture was used for spread plating on LB plates with 100µg/ml ampicillin and incubated overnight at 37ºC.

The second electroporation for both MG1655 and C29 strains, already containing pKD46, was identical to the first transformation procedure except that the amplified PCR products were electroporated into the strains. In addition, transformants were plated on LB plates with 50µg/ml kanamycin.

During the transformation process, LB media was used to substitute SOB media. Although the transformation protocol included with the BioRad MicroPulser instructed the use of SOB media, it was determined that LB broth could be used as an equivalent medium.

β-galactosidase Assay. The measurement of β-galactosidase activity in MG1655 cells was performed using a variant of the protocols outlined in Jaeger's original experiment (6). Ten randomly selected colonies were chosen from a kanamycin X-gal LB plate and inoculated in 5ml of LB broth with 50µg/ml kanamycin, and 5ml of LB broth with 50µg/ml kanamycin and 1mM IPTG. A wild-type MG1655 clone was also selected and grown on both induced and non-induced media. All cell suspensions were incubated overnight at 37ºC with shaking. Two hundred microlitres of toluene was added to each tube and vortexed. After allowing the solution to separate into separate phases, 100µl of the cell suspensions were transferred to a separate reaction tubes containing 300µl of sterile distilled water and 1.2ml of tris buffer. The enzymatic reaction began once 200µl of 5nM ONPG was added. The reactions were allowed to proceed in a 37ºC water bath, and stopped upon the addition of 2ml 0.6M sodium carbonate. The absorbance of each tube was then measured at a wavelength of 420nm.

RESULTS

Initial selection of the C29 strain carrying the pKD46 plasmid was carried out using ampicillin LB plates. Successful transformation of the parental MG1655 strain with pKD46 was demonstrated by the high number of transformants that grew on the LB ampicillin plates.

The second round of transformation, involving both modified MG1655 and modified C29 strains, produced successful transformants as well (MG1655: WL051-WL054; C29: WL055-WL058). Both strains were transformed with the kanamycin-resistance cassette which was amplified by PCR. When plated on kanamycin LB plates, both transformed strains had demonstrated growth. Since the kanamycin-resistance cassettes are not self-sustainable, it is reasonably safe to assume that there was successful integration of the cassette into the host chromosomes.

PCR amplification of the kanamycin-resistance cassette was done using two sets of primers. The first primer set, designed for *pho*A-targeting resulted in a PCR product with an approximate size of 900bp (Fig. 3). This size did not match the expected size of 1251bp. The second set of primers, designed for *lac*I-targeting, gave a PCR product that appears identical to the other one at 900bp. This fragment closely matched the expected size of 891bp.

Optimization of PCR amplification of the kanamycin-resistance cassette determined that a suitable annealing temperature was anywhere between 41.9 and 55.9ºC (Fig. 3). The amount of $MgCl_2$ used in the PCR mix was also varied to study its effect on the amount of PCR product. In addition to the original concentration of 1.5mM $MgCl_2$, a final concentration of 3.0mM $MgCl_2$ was also tested. However, after running the PCR products resulting from an $MgCl_2$ concentration of 1.5mM, it was determined that there was sufficient PCR product to carry out the transformation of both MG1655 and C29. Thus, the PCR products resulting from an $MgCl_2$ concentration of 3.0mM were not tested on gel electrophoresis.

A screening process on LB agar with 5-bromo-4-chloro-3-indolyl phosphate was used to determine whether or not the alkaline phosphatase gene was deactivated in the mutagenized C29 isolates. After overnight incubation, all of the colonies appeared blue, suggesting that alkaline phosphatase was still active.

A similar screening process was carried out on LB agar to determine MG1655's ability to constitutively express β-galactosidase in the presence and absence of IPTG. Preliminary results showed the presence of blue colonies of similar intensities in both induced and non-induced conditions. This initial observation suggested that induction did not make a difference in β-galactosidase production. However, the results from the actual β-galactosidase assay showed that this assertion was incorrect.

The β-galactosidase assay performed on MG1655 (Fig. 4) clearly showed that in the absence of IPTG, β-galactosidase production was severely limited. For most of the samples, including the wild-type MG1655, IPTG induction resulted in β-galactosidase production approximately 10X higher than the basal level. This suggested that *lac*I was not properly deactivated and was still functional. The data obtained for sample 6 should be ignored due to a human error discovered during the β-galactosidase test where induced and non-

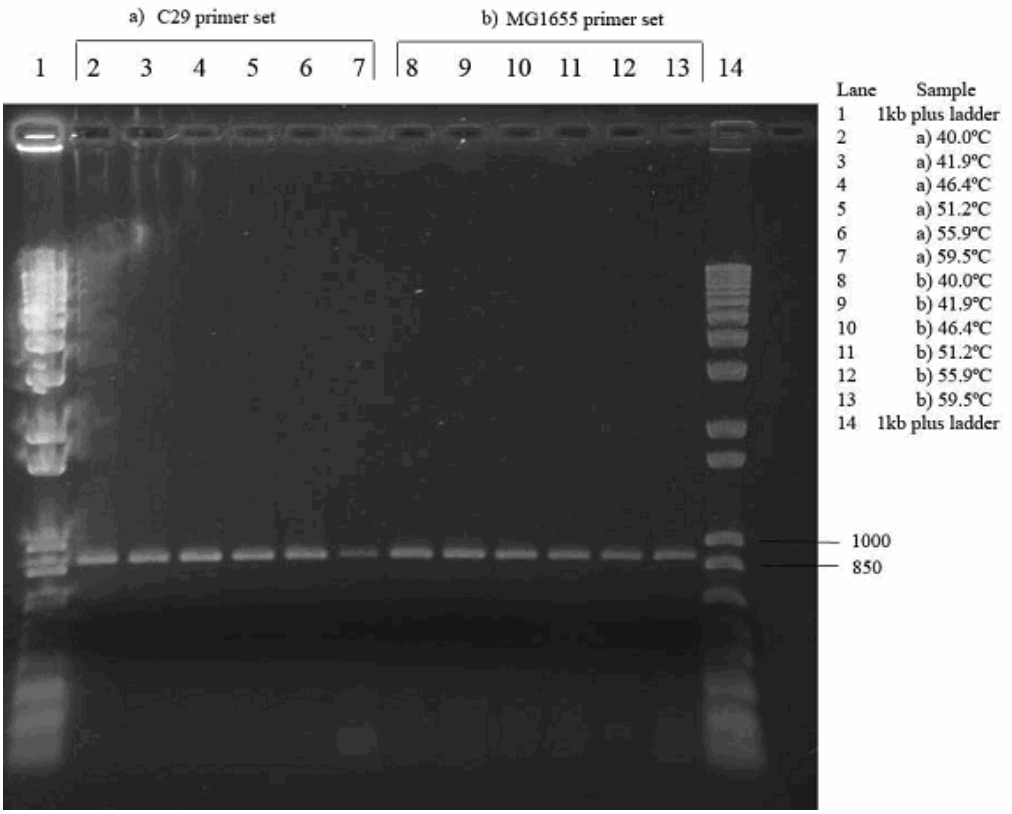

FIG. 3 Gel electrophoresis of amplified kanamycin-resistance cassettes for both MG1655 and C29. a) refers to fragments amplified using the *pho*A-targeting primer set under varying temperatures while b) refers to those amplified by the *lac*I-targeting primer set under varying temperatures. Lanes 1 and 14 are the Invitrogen 1kb plus molecular weight standards.

induced conditions were switched, resulting in an inconsistency that can be seen in figure 4.

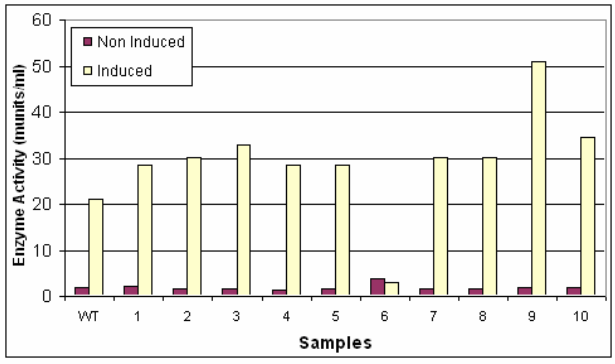

FIG. 4 β-galactosidase assay of transformed MG1655 cell cultures in the presence and absence of IPTG. The numbers for the various samples represent the different clones. The WT is the wild-type control. It should be noted that the data from sample 6 is not valid and should be ignored.

DISCUSSION

While neither of the targeted genes was successfully disrupted, the results from this experiment support the hypothesis that the λ-red recombinase system was not gene or strain-specific. In each case (MG1655 and C29) the λ-red recombinase system was unable to properly deactivate *lac*I or *pho*A genes. While specific deactivation did not occur, it was demonstrated that integration of the kanamycin-resistance cassettes did occur somewhere in the host. This is known because the kanamycin fragments were not self-sustainable and required integration into the host chromosome to allow for kanamycin-resistance, which was demonstrated following the transformation process. Integration of the kanamycin-resistance cassette into pKD46 is also unlikely since it is a low copy number plasmid (4). The results obtained from this study closely resembled those obtained by Jaeger *et al.* (6), Woo (12), and Cheema (3). These observations suggest that the inability to disrupt *lac*I in *E.coli* C29 strains was not specific for those studies and that a more wide-spread problem could be the cause.

One aspect that may have been overlooked in this study and previous studies was the specificity of the primers as a whole. While it was shown that each half (15nts) of each of the primers was specific only for their respective targets, the specificity of the entire 30nt

primer was not tested against the chromosomes of the targeted strains (10). It is possible that certain regions of the combined 30nt primer are homologous to other regions of the host chromosome. This would allow for site-specific integration of the kanamycin-resistance cassette at a region of the chromosome not yet accounted for (10).

One possible reason that deactivation of alkaline phosphatase did not occur is if an incorrect PCR fragment was used. As noted earlier, the PCR fragments obtained using the *lac*I-targeting primer set was around 350 base pairs smaller than the expected size. From analyzing Figure 3, there is a possibility that the fragments amplified using the *lac*I-targeting primer set is identical to those amplified with the *pho*A-targeting primer set. This could be due to a mix up in the use of primers where the *lac*I-targeting primer set was mistakenly used for both experiments. Given this to be the case, it would be expected that deactivation of the *pho*A gene could not possibly occur because the amplified gene was incorrect. However, while deactivation indeed did not occur, integration of the fragment still took place. Non-specific integration by the λ-red system is one possibility and is supported by a previous study where it was shown that integration of the kanamycin-resistance cassette did not occur directly at the *lac*I gene (8).

In regards to the original experiment carried out by Jaeger *et al.* (6), it was hypothesized that there was another *lac*I gene present in the C29 genome. This theory assumed that integration of the kanamycin-resistance cassette only occurred at one of the *lac*I sites, thus β-galactosidase production was still being regulated. A similar theory can not be stated for the results obtained from modifying MG1655 in this study since it is known that there is only one copy of the *lac*I gene available. It is important to note that this result certainly can not disprove the possibility of a second copy of *lac*I in the C29 strain that Jaeger *et al.* (6) and others had studied. However, it does raise the possibility that the λ-red recombinase system is not working for a more general reason applying to all of the studies conducted thus far including the original experiment performed by *Jaeger et al*. (6).

The results obtained from Jaeger *et al*. (6) and others certainly did not discredit the λ-red recombinase system and its ability for site-directed mutagenesis. Researchers such as Datsenko have been successful in utilizing the λ-red system to accurately target and disrupt 40 different genes (4). Though both studies utilize the λ-red system for site-directed mutagenesis, many differences exist between Datsenko's (4) experiment and this experiment. For their bacterial strains, Datsenko used a range of E. coli K-12 strain BD792 derivatives: BW25113, BW25993, and BW25141 (4). One notable difference in the method used by Datsenko (4) was the presence of FRT sites flanking the antibiotic gene, allowing for removal of the antibiotic gene through the use of the flp recombinase. However, applying this method would to our study would not likely cause successful integration since the FRT sites do not assist in the targeting of the kanamycin-resistance cassette. Another aspect of Datsenko's (4) experiment that is different from this study is the length of primers used in generating the PCR products. While this study only utilized primers 30nt in length, Datsenko generated primers 56-70nt long with 36-50nt specifically targeting the gene of interest (4). An increased primer length increases the likelihood of getting primer dimers and hairpin loop formation. However, a longer primer also allows for more pairing between the primer and the template DNA, thus increasing the stability of this interaction. Therefore, it is possible that increasing the length of the extensions homologous to *lac*I and *pho*A in the respective primers will increase the chances of successful integration.

FUTURE EXPERIMENTS

PCR amplification of the kanamycin-resistance cassette using both *lac*I and *pho*A-targeting sets of primers should be carried out and analyzed on gel electrophoresis. This should be done to determine why the PCR product size obtained using the *pho*A-targeting primer set did not match the expected size. Repeating this small test will quickly determine if the discrepancy between the obtained fragment size and the actual fragment size was due to human error. If it is determined that both primer sets still produce fragments with similar band sizes, it is likely that the primers used to target *pho*A were incorrectly made. A new set of *pho*A-targeting primers should be synthesized if this is the case. This should be followed by re-amplification of the Kanamycin-resistance cassette using the newly synthesized *pho*A-targeting primers.

From both of the studies, it is reasonable to believe that integration of the kanamycin-resistance cassette occurred, but did not properly disable their respective targets. Therefore, another experiment to be carried out for both studies should be chromosomal-walking to determine where the kanamycin-resistance cassette was integrated. A similar experiment was attempted on the original C29 strain constructed by Jaeger *et al*. but was not completed in time (2).

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