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Successful Application of the λ-Red Recombinase System to Inactivate *lacI* in *Escherichia coli* C29, BW25993 and MG1655

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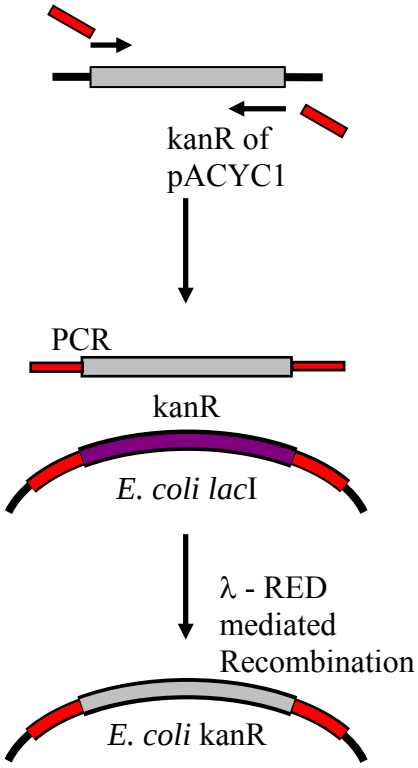
The Lambda Red Recombinase system (λ-Red) is a simple and efficient site-specific mutagenesis method. This study details the successful utilization of this method to inactivate *lacI* in *Escherichia coli* strains C29, BW25993, and MG1655 in the context of the Microbiology 421 laboratory. Previous studies performed in this setting have attempted to use this method to knockout the *lacI* gene from *E. coli* C29, thereby disrupting the repressor activities of the *lacI* gene product allowing for constitutive expression of β-galactosidase. Potential explanations for the lack of success in the past include mis-annotated primer design, sub-optimal L-arabinose induction of the recombinase genes, and low recombination frequency. In this study a PCR product containing a kanamycin resistance gene was produced using primers that allow for genotypic identification of the inserted construct, flanked with homologous sequence found upstream and downstream of the *lacI* gene. This product was transformed into all three *E.coli* strains containing the recombinase plasmid. Selection for kanamycin resistance confirmed the successful transformants. Preliminary evidence shows that deletion of *lacI* was successful in all three strains, and conclusive enzyme assay data supported this in both *E. coli* C29 and BW25993 strains. This study provides the first evidence of successful use of the λ-Red recombinase system in the Microbiology 421 laboratory. This work provides a basis for future studies; the λ-Red system is a rapid and specific method for gene interruption and this work is an important first step in its practical application for gene-function analysis.

The Lambda Red Recombinase system (λ-Red) is a site-specific mutagenesis method developed by Datsenko and Wanner (5). It is used to disrupt chromosomal genes in *Escherichia coli* with an antibiotic resistance marker. In this system, PCR primers flanked with homologous sequence found upstream and downstream of the target gene are used to provide the homology to the targeted genes. This system is a very powerful tool; in fact it has been used to systematically delete all non-essential genes in *Escherichia coli* K12 (2). Several attempts have been made in the Microbiology 421 laboratory to disrupt the *lacI* gene in *E. coli* C29 by replacing it with a kanamycin resistance gene (*kanR)* using this method (see FIG. 1). To date, all construction attempts have been unsuccessful due to errors while using the recombination system (1, 3, 9). Bepple *et al.* (unpublished data) noted these mistakes and, although they corrected them, were still unable to construct the *lacI* deletion. They have hypothesized that this could be due to the introduction of non-methylated DNA into an *E. coli* strain (C29) whose genome exhibits DNA methylation as non-methylated DNA may be targeted for degradation.

Problems in cloning and transformation can arise when strains with functional Type I restriction modification systems are used as recipients of unmethylated DNA. Type I restriction endonucleases are composed of three subunits, HsdR, HsdM, and HsdS; the trimeric complex cleaves unmethylated DNA sequences 5′-AAC[N_6]GTGC-3′(5) (10). HsdR, while not required for methylation activity, is absolutely required for endonuclease activity at this target sequence. Thus, a strain mutant in *hsdR* is a suitable endonuclease-deficient control, allowing for entry of unmethylated DNA into the host bacterium (10).

In this investigation, both the *hsdR+* and *hsdR-* strains used in the original work with the λ-Red system by Datsenko and Wanner, MG1655 and BW25993, respectively (5), were used in parallel with the *hsdR+* strain C29 that has been used in previous attempts within this laboratory (1, 3, 9). By using all three, the effect of methylation and endonuclease activity could be tested since the *hsdR+* strain MG1655 served as a positive control for our PCR product, and the *hsdR-* strain, BW25993 (otherwise very similar to MG1655) acted to test the effect of endonuclease activity on the system. Using C29 served as a control for comparison with previous, unsuccessful attempts (1, 3, 9).

This experiment was designed to test whether previously unsuccessful attempts to use the λ-Red



system in C29 were due entirely to technical problems such as primer design, or whether additional characteristics of C29 such as *hsd* were responsible for the failures. This was done by testing two strains used in the original work of Datsenko and Wanner (5) in parallel with C29. The use of positive controls were intended to differentiate failure due to technical errors from failure due to the strain characteristics of C29 and provide an indication of whether *hsdR* plays an important role in the successful use of the λ-Red system.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* strains DH5α/pACYC177, MG1655/pKD46, C29/pKD46 were obtained from the University of British Columbia's MICB 421 collection and *E. coli* strain BW25993 was obtained from the Coli Genetic Stock Center at Yale University (Table 1). *E. coli* strain DH5α was used to obtain the pACYC177 plasmid and was grown at 37ºC in Luria-Bertani (LB) medium (1 % tryptone, 0.5 % yeast extract, and 1 % NaCl) supplemented with 50 µg/mL kanamycin (Sigma, St. Louis, MO). *E. coli* strain MG1655/pKD46 was used to obtain the pKD46 plasmid and was grown at 30ºC in LB broth supplemented with 100 µg/mL ampicillin (Sigma, St. Louis, MO). *E. coli* strain BW25993 was grown in LB broth at 37ºC prior to the insertion of the pKD46 plasmid (FIG. 2). *E. coli* strains C29/pKD46, MG1655/pKD46, and BW25993/pKD46 were grown at 30ºC in LB broth supplemented with 100 µg/mL ampicillin.. For the β-galactosidase enzyme assay, C29/pKD46, MG1655/pKD46, and BW25993/pKD46 and one putative transformant for each strain were cultured at 37ºC in M9 minimal media (M9 salts: 0.6 % Na2HPO4, 0.3 % KH2PO4, 0.05 % NaCl and 1 % NH4Cl; supplemented with 1 mM MgSO4•7H20, 0.4 % glycerol, 0.5 % Bacto Tryptone, and 0.25% Bacto Yeast) and incubated with mild aeration.

TABLE 1. *Escherichia coli* strains used
*pKD46 confers the following phenotypic characteristics: *repA101(ts); araBp-gam-bet-exo; oriR101; bla(ApR)*

***E. coli* Strains and Plasmids**	**Strain Characteristics**	**Reference**	**Source**
MG1655/pKD46*	*LAM-; rph-1*	CGSC# 7669	MICB 421 collection
C29/pKD46*	Hfr PO 12.20' CW *tonA22, phoR19* (constit) *ompF627* ($T2^R$) *fad701* ($T2^R$) *relA1 pit-10 spoT1 rrnB-2 mcrB1 creC510*	CGSC#4936	MICB 421 collection
BW25993	*DE(araD-araB)567; LAM-; rph-1; DE(rhaD-rhaB)568; hsdR514*	CGSC#7639	Yale University Coli Genome Collection
DH5α/pACYC177	*DE(argF-lac)169; $\phi80$dlacZ58(M15); glnV44(AS); LAM-; rfbC1; gyrA96(NalR); recA1; endA1; spoT1; thi-1; hsdR17*	13	MICB 421 collection

Plasmids. pACYC177 was used as a template for PCR amplification of the kanamycin resistance gene. pACYC177 is a commonly used multi-copy cloning vector (4), 3940 bp in length, and encodes for both kanamycin and ampicillin resistance. Plasmid pKD46 (5) is a 6329 bp low copy number plasmid which encodes the Red genes (γ, β, *exo*) under the control of an arabinose-regulated promoter, *araC-ParaB.* pKD46 is temperature sensitive for easy curing at high temperature (42ºC), and expresses ampicillin resistance as a selection marker. Plasmids pACYC177 and pKD46 were isolated from *E. coli* strains MG1655/pKD46 and DH5α/pACYC177. O/N cultures were performed in 3 mL LB medium supplemented with 100 µg/mL ampicillin or 50 µg/mL kanamycin, respectively, and incubated with mild aeration at 30ºC. Plasmids were isolated using PureLink Quick Plasmid MiniPrep Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

PCR Amplifications. The following reagents were used to amplify the kanamycin resistance gene and confirm upstream and downstream junctions in recombinants: 1X PCR buffer, 0.2 mM dNTP, 1mM) MgCl , 0.4 uM forward primer (see Table 2), 0.4 uM reverse primer (see Table 2), 1.25 U Taq polymerase (Fermentas, Burlington, Ontario, Canada), and distilled H2O to a final volume of 50 µL. The PCR reaction conditions used to

amplify *kanR* were: an initial denaturation 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. The PCR reaction conditions used to amplify the junction were: an initial denaturation of 7 minutes at 95°C; 34 cycles of 45 seconds at 94°C, 30 seconds at 60°C, and 60 seconds at 72°C; followed by a final extension of 10 minutes at 72°C.

TABLE 2. Primer sequences

Primer ID	Description	Nucleotide Sequence (5' to 3')
#1	*kanR* for recombination (forward)	TAGCGCCCGGAAGA GAGTCAATTCAGGG TGGTGAATGTGagcca tattcaacggg†
#2	*kanR* for recombination (reverse)	GCCTAATGAGTGAG CTAACTCACATTAA TTGCGTTGCGCttagaa aaactcatcgac†
#3	Upstream junction of *kanR* for confirmation (forward; coding)	TTGACACCATCGAA TGGCGC
#4	Upstream junction of *kanR* for confirmation (reverse; non-coding; Kan internal)	TTGGCAACGCTACC TTTGCC
#5	Downstream junction of *kanR* for confirmation (forward; coding; Kan internal)	GACCGATACCAGGA TCTTGCC
#6	Downstream junction of *kanR* for confirmation (reverse; non-coding)	AACGACGGCCAGTG AATCCG

†Capital letters correspond to external overlap with *lacI*, lowercase letters indicate internal overlap with *kanR*

Agarose Gel Isolation. Linear PCR product was isolated from a 1.5 % agarose gel using the GenElute gel extraction kit (Sigma, St. Louis, MO) according to the manufacturer's protocol.

Preparation of electrocompetent cells. For preparation of electrocompetent BW25993, 5 ml LB medium was inoculated with a well-isolated colony and incubated overnight at 30°C. For preparation of electrocompetent MG1655/pKD46, C29/pKD46, and BW25993/pKD46, 5 ml LB supplemented with 100 µg/mL ampicillin was inoculated with a well-isolated colony and incubated overnight at 30°C. The next day, 1 mL of the overnight culture was added to 100 ml SOB (2 % tryptone, 0.5 % yeast extract, 10 mM NaCl, 12.5 mM KCl, and 10 mM MgCl2) supplemented with 20 mM MgSO4 (BW25993) or SOB supplemented with 20 mM MgSO4, 100 µg/mL ampicillin and 1 mM L-arabinose (MG1655/pKD46, C29/pKD46, and BW25993/pKD46). Cultures were incubated at 30°C while shaking at 200 rpm until an $OD_{600} \sim 0.4$. Culture was chilled on ice for 15 minutes, then transferred to a sterile centrifuge tube and centrifuged at 4000 rpm for 5 min at 4°C. Cell pellet was washed once in 100 mL ice cold 10% glycerol then once in 50mL ice cold 10% glycerol. Following the wash steps the pellet was resuspended in 1mL ice cold glycerol and distributed into 50 µL aliquots. Aliquots were flash frozen in a dry ice/ethanol bath and stored at -80°C.

Transformation. An aliquot of prepared electrocompetent cells was thawed on ice. In a microfuge tube, 50 µL of electrocompetent cells were mixed with 2 µL of DNA and incubated on ice for 1 minute. The cells were then transferred to a chilled 0.2 cm electroporation cuvette (BioRad, CA, USA) and pulsed once at 2.5 kV with a BioRad GenePulser(BioRad MicroPulser, BioRad, CA, USA). Immediately 1 mL of LB medium was added to the cuvette and the suspension was then transferred to a 1.5 mL microfuge tube and incubated at 37°C for 1 hour. After recovery, 10 µL and a 100 µL resuspension of the remaining transformants were then spread on LB agar supplemented with 100 µg/mL ampicillin (BW25993) or LB agar supplemented with 50 µg/mL kanamycin and 80 µL of a 20 mg/mL stock of X-Gal (MG1655/pKD46, C29/pKD46, and BW25993/pKD46).

Cell Disruption. C29/pKD46, MG1655/pKD46, and BW25993/pKD46 and one putative transformant for each strain were cultured overnight in supplemented M9 medium at 37°C, 200 RPM. A 1:15 dilution of overnight culture was used to inoculate fresh M9 medium, and cells were grown for 4 hours at 37°C, 200 RPM. Cells were then spun at 7500 X g for 10 minutes, and the pellet was resuspended in 1.1 mL TM buffer (10 mM Tris (pH 8.0), 0.1 mM magnesium chloride) and transferred to 1.5 mL FastPrep microfuge tubes containing 0.1 mL of 0.1 mm glass beads. Cells were agitated for three successive 30 second bursts at a power setting of 6 on the Fast Prep Cell Disrupter (Bio 101/Savant Instruments, Holbrook, NY). Following disruption, 0.05 mL of a stock solution containing 300 µg/mL deoxyribonuclease and 300 µg/mL ribonuclease was added to each tube, and samples were incubated at 30°C for 15 minutes. After the nucleic acid digest step, samples were chilled on ice for 4 minutes, and spun at 14,000 X g for 5 minutes. Next, samples were filtered into 1.5 mL microfuge tubes through 0.2 µm pore filters.

Galactosidase Enzyme Activity Assay. For the galactosidase enzyme assay, 25 µL of sample was added to 1.275 mL of TM buffer and 0.2 mL of 5 mM ONPG pre-warmed to 30°C, mixed immediately, and incubated at 30°C. When sufficient yellow color developed, 2 mL of 0.6 M sodium carbonate was added to stop the reaction. Absorbance was measured using a Spectronic 20 spectrophotometer set at 420 nm.

Bradford Protein Assay. The Bradford assay was used to measure total protein in the cell lysates. To do the assay, 2 mL of Bradford dye reagent (Biorad) was added to 1 mL of diluted sample or standard solution, vortexed, and incubated at room temperature for 10 minutes. A standard curve was generated using bovine serum albumin (Sigma, St. Louis, MO). Standards were prepared from a 1 mg/mL stock solution using distilled water for final concentrations of 0, 5, 10, 20, 30, 45, 60, and 80 µg/mL. Samples were diluted 1/100 in distilled water. Absorbance was measured using a Spectronic 20 spectrophotometer set at 595 nm, blanked using the 0 µg/mL standard described above.

RESULTS

The BW25993 *E. coli* strain was successfully transformed with pKD46 and the temperature-sensitive ampicillin resistance phenotype confirmed by growth at 30°C and absence of growth at 42°C (data not shown). MG1655/pKD46 and C29/pKD46 were also phenotypically tested to confirm the maintenance of the plasmid. The pACYC177 plasmid was isolated from DH5α cells at a final concentration of 51 µg/mL. The plasmid identity was confirmed by gel visualization (data

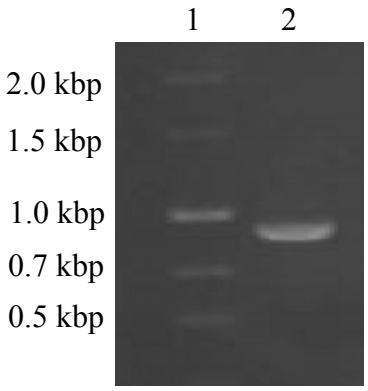


FIG. 3. Gel isolated PCR product (lane 2) used for *lacI* replacement in *E. coli* BW25993, MG1655 and C29. The PCR product was electrophoresed in a 1.5% agaraose gel in TAE

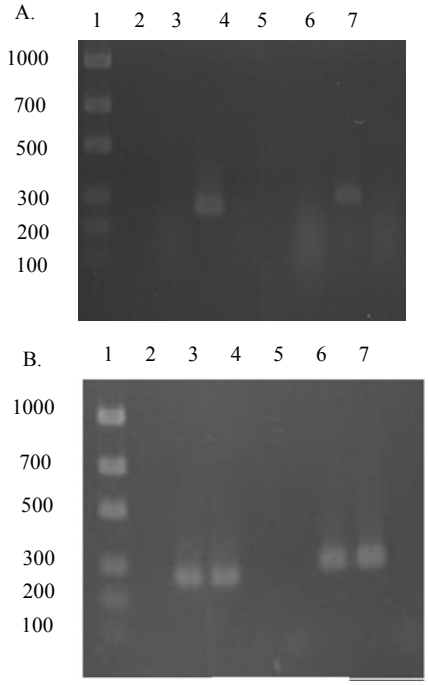


FIG. 4. Amplification of recombinant junctions from *E. coli* BW25993 Δ*lacI*, MG1655 Δ*lacI*, and C29 Δ*lacI* visualized in a 1.5% agarose gel electrophoresed in TAE buffer at 100V for 1hr. (A) Upstream junction (BW25993) shown in lane 4 (265 bp) and downstream junction (BW25993) shown in lane 8 (289 bp) in comparison to pACYC177 (lanes 2 and 6), BW25993/pKD46 (lanes 3 and 7) and No DNA control (lanes 5 and 9). (B) Upstream junction shown in lanes 3 (MG1655) and 4 (C29) (265 bp) and downstream junction shown in lanes 7 (MG1655) and 8 (C29) (289 bp) in comparison to pACYC177 (lanes 2 and 6) and No DNA control (lanes 5&9).

not shown) and PCR amplification of the kanamycin resistance gene. The *kanR* gene was amplified using Primer #1 and Primer #2 (Table 2), and the expected product of 891 bp observed and successfully purified using gel isolation to a final concentration of approximately 30 ng/μL (FIG. 3).Electrocompetent BW25993 / pKD46, MG1655 / pKD46, C29/pKD46 *E. coli* cells were prepared. Transformation by electroporation of all strains was successful, with time constants of 5.7 ms, 5.8 ms, and 5.9 ms respectively. Although transformations were successful in producing at least a single recombinant, there were a low number of recombinants (less than one in 10^6) based on the number of blue colonies showing constitutive expression of *lacZ* on LB plus kanamycin plates with X-gal (data not shown). Viable cell counts would be necessary to determine transformation efficiency more precisely.

Two separate lines of evidence confirmed the successful deletion of *lacI* in the three tested strains: PCR amplification of the junction and constitutive expression of *lacZ*. FIG. 4a displays the results of PCR amplification of the upstream and downstream junction for BW25993, while FIG. 4b displays the results of MG1655 and C29; products were of the expected size (265 bp for upstream and 289 bp for downstream).

The results of a discontinuous enzyme assay comparing β-galactosidase expression for parental and recombinant cultures of the three tested strains are displayed in Table 3. Once normalized for total protein content, two of the strains, BW25993 and C29, had notably elevated β-galactosidase expression (in the absence of inducer) in the recombinant compared to wild-type strains, consistent with successful deletion of the *lacI* repressor in these strains. The third strain, MG1655, did not show notably elevated β-galactosidase activity in the discontinuous enzyme assay performed. Subsequent re-plating of the MG1655 used for the enzyme assay revealed a mixed population of cells, heterogonous for constitutive expression of *lacZ*. Due to time constraints, a discontinuous enzyme assay was not performed on re-isolated colonies.

TABLE 3. Normalized β-galactosidase activity observed in the various strains of *E. coli*.

Sample Identification	Normalized Enzyme Activity (miliunits/mg)
BW25993/pKD46*	4
BW25993/Δ*lacI*	526
MG1655/pKD46*	3
MG1655/Δ*lacI*	2
C29/pKD46*	2
C29/Δ*lacI*	134

*Cells were grown at 37°C. pKD46 is a temperature-sensitive plasmid. Growth of transformants at 37°C would have cured most pKD46, although ampicillin sensitivity of the transformants was not tested.

DISCUSSION

This study provides the first evidence of successful use of the λ-Red recombinase system in the Microbiology 421 laboratory. Preliminary evidence shows that deletion of *lacI* was successful in all three strains, and conclusive enzyme assay data supported this in both *E. coli* C29 and *E. coli* BW25993 strains. Subsequent examination of the third strain, *E. coli* MG1655, revealed that the culture was not pure, which may account for the low level of constitutive expression of β-galactosidase observed in the enzyme assay (see Table 3). PCR amplification of the *kanR* junction using an isolated colony from this purification constitutively expressing *lacZ* (based on growth on X-gal plates) was successful indicating that the recombination was indeed successful in MG1655, although additional evidence from an enzyme assay would be required to conclusively determine the successful inactivation of *lacI* in this strain.

One contributing factor for the success of this attempt at using the λ-Red system may have been the improved primer design. The λ-Red system is based on enzyme mediated homologous recombination (5). Upon review of previous attempts to disrupt the *lacI* in *E. coli* C29 (Bepple *et al.,* unpublished data, 3), it was noticed that the kanamycin resistance gene was mis-annotated in the pACYC177 sequence file (13). Comparison of the pACYC177 sequence to the parent sequence of the Tn903 kanamycin resistance gene (12) revealed that the start codon was 2 bp downstream of the indicated position and that the stop codon was 5 bp downstream of the indicated position. This meant that previous groups did not amplify the whole gene. Incomplete gene amplification may have affected the translation and expression of the gene product. Without expression of the resistance marker all Δ*lacI* recombinants would fail to grow under kanamycin selection; this may account for the lack of recombinants in previous studies.

In this study, we redesigned the primers based on the properly annotated sequence. In addition, because this project entailed a gene replacement of the *lacI* with the kanamycin resistance gene, the native promoter of the *kanR* was not amplified. This meant that expression was under the control of the *lacI* promoter. In order to maintain any spatially important transcriptional controls, the start or stop codon of the *kanR* were fused to homologous sequence directly upstream or downstream of the *lacI* start or stop codon. Other groups did not maintain the sequence spacing between the promoter sequence and the start codon by adding sequence (Bepple *et al.*, unpublished data). This may have affected transcription initiation or termination and may account for the lack of *lacI* recombinants in previous studies.

Another important factor leading to the successful *lacI* disruption using the λ-Red system may have been the increased concentration of L-arabinose that we used to induce the expression of recombination proteins from pKD46. Previous studies had used 1 mM L-arabinose as described by Datsenko and Wanner (5) but in this study we used 10 mM L-arabinose. Datsenko and Wanner noted that for *E. coli* MG1655 that is not Δ*araBAD* they found more recombinants in the presence of 10mM L-arabinose than 1mM L-arabinose (5). Gust *et al* (7) also used 10 mM L-arabinose to induce recombination genes. An increase in L-arabinose concentration may have helped to increase the efficiency of recombination by more fully inducing gene transcription from pKD46.

A third potential explanation of the difference between this study and previous studies is that despite plating a large number of cells following electroporation, we still saw only a small number of putative recombinants. This was possibly a result of the electroporation conditions used in this study, which resulted in time of 5.7 to 5.9 ms, much higher than the ideal range which is below 4.6 ms; high time constants may result in low cell survival as the discharge occurred too slowly (6, 11). With this in mind, groups that plated fewer cells (1) may not have observed any recombinants at all if they had similar recombination frequencies. The number of recombinants is too low to assess the effect of *hsd* on the recombination frequency, however the occurrence of recombinants in the hsd^+ and the hsd strain indicates that hsd^+ is not an absolute barrier to recombination.

This work provides a basis for future studies as the λ-red system is a rapid and specific method for gene interruption. Creation of a working protocol for the use of this system under these laboratory conditions represents an important first step in its practical application for gene-function analysis in Microbiology 421. In addition, demonstrating that this recombination system can be used in both *hsdr+* and *hsdR-* bacterial strains now allows future Microbiology 421 groups to go on to create new gene deletions in other *E. coli* strains, or even establish this system in other types of bacteria, such as antibiotic producing *Streptomyces*, in which gene disruption using the λ-Red system has been reported in the literature (8).

FUTURE EXPERIMENTS

Continuation of this work should examine our initial hypothesis about the effect of *hsdR* on the λ-Red system. This work has demonstrated that both *hsdR*+ and *hsdR*- strains can be used as a background for this system, however as recombination efficiencies were not specifically calculated, it could not be determined whether this has an effect on the efficiency of recombination. One other factor that may influence the recombination efficiency of BW25993 is the deletion of the arabinose operon in this strain (see Table 1). The absence of an endogenous arabinose operon may improve induction, since there would be no competition for the arabinose operon on pKD46. In order for a comparison of recombination efficiencies to be feasible, recombination efficiencies need to be higher than we achieved here. Future groups might need to look at optimizing the preparation of electrocompetent cells and electroporation conditions.

Although our enzyme assay allowed us to compare the putative transformants with the parental strains by observing the disruption of *lacI* based on β-galactosidase activity in the absence of any inducer, an additional confirmation of successful disruption in putative transformants could test for β-galactosidase enzyme activity in presence of IPTG. If expression level is unchanged in the presence and absence of inducer, then this final step would confirm that the observed phenotype was due to specific interruption of *lacI* by the λ-Red system.

Based on issues we encountered with impurity of a putative transformant for MG1655 in the β-galactosidase enzyme assay, we recommend that future experiments perform additional colony purification steps to ensure purity of transformants prior to testing for successful disruption.

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