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Examinations and Continued Development of the Lambda Red Recombinase Mediated Genomic Knockout of *lacI* in *Escherichia coli* C29

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The Lamda Red Recombinase system is a site-specific mutagenesis method allowing for the swapping of targeted genes in the bacterial chromosome with an antibiotic selection factor. This study, along with previous studies, strived to employ this method to knockout the lacI gene from the Escherichia coli C29 host strain in order to remove the repressor activities of the lacI gene product allowing for constitutive expression of β-galactosidase. Previous attempts at disrupting lacI in this strain may have failed due to partial or complete curing of the temperature sensitive recombinase plasmid pKD46 with growth at 37°C, as well as sub-optimal and inconsistent L-arabinose induction of the recombinase genes. In this study, a PCR product containing kanamycin was produced using primers which allow for genotypic identification of the inserted construct. This product was transformed into C29 cells containing the recombinase plasmid, with selection for kanamycin resistance revealing transformants. Examination of kanamycin resistant colonies using an enzyme plate assay indicated tight regulation of the β-galactosidase enzyme via lacI repression and as such, unsuccessful recombination using the Lambda Red Recombinase method in C29 cells. From these results it was concluded that the influence of growth at the required 30°C and L-arabinose induction, were insufficient to promote disruption of the lacI gene. Additionally, the presence of a short linking region within the PCR produced insertion construct compared to the previous constructs would allow improved identification of the isolates containing the construct.

Previously, a number of studies (1, 2, 7, 10, 14) have attempted to utilize the Lambda Red Recombinase (LRR) mediated genomic knockout method developed by Datsenko and Wanner (8) in order to remove the repressor activity of lacI within the Escherichia coli C29 strain. These previous attempts strived to isolate cells with constitutive β-galactosidase (β-gal) expression due to the loss of the lacI repressor. To accomplish this an insertion construct composed of the gene conferring kanamycin resistance amplified from the pACYC177 plasmid was produced within a linear PCR produced construct with terminal regions homologous to the targeted insertion site of the genomic lacI gene. Despite using this construct, isolated kanamycin resistant colonies assayed for β-gal expression were found to be strongly regulated as if there was ineffective disruption of the lacI gene within the C29 strain. Given the robust kanamycin resistance, and well regulated β-gal expression seen in previous studies, questions regarding the origins and location of the kanamycin resistance seen in these isolates were posed.

The main technique used to mediate the genomic disruption of the lacI gene is the LRR system (6, 10). This system uses the same site-specific homologous recombination aspects of lambda viral genes to stimulate a targeted insertion of a linear PCR product gene sequence into the host bacterial genome (6). The LRR system, seen in Figure 1a, is contained within the pKD46 plasmid contains the recombinase genes γ, β, and exo under the control of an L-arabinose inducible promoter P_{araB} (6). The γ gene yields the protein Gam, an inhibitor of the bacterial RecBCD exonucleaseV enzyme, thus protecting incoming DNA from degradation. The Bet and Exo proteins, from the β and exo genes respectively, promote integration of the exogenously added DNA fragment targeted to the bacterial genome in a site-specific manner. Under growth conditions containing L-arabinose, the P_{araB} promoter is induced and the recombinase genes are expressed.

In this study, numerous factors known to influence the LRR mediated site-specific mutagenesis were applied to C29 cells containing the pKD46 plasmid. Following growth at 30°C in the presence of 1mM L-arabinose in a glucose-free growth medium, cells electroporated with a novel insertion construct, which allows for identification of isolates containing the insertion construct, a number of kanamycin resistant colonies were isolated and examined for

regulation of β-gal expression. We hypothesised that these factors would result in the successful isolation of kanamycin resistant colonies that demonstrated loss of repression of β-gal expression due to lacI disruption by the insertion construct. However, while kanamycin resistant isolates were obtained, they failed to have derepressed β-gal expression.

MATERIALS AND METHODS

Bacterial strains and growth conditions. E. coli strain DH5α used to obtain the pACYC177 plasmid (6) was grown at 37ºC in Luria-Bertani (LB) broth (1%w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl) supplemented with 50 µg/mL kanamycin. E. coli strain MG1655 used to obtain the pKD46 plasmid was grown at 30ºC in LB broth supplemented with 100 µg/mL ampicillin. E. coli strain C29 transformed with the isolated pKD46 plasmid was grown in LB broth at 37ºC prior to the insertion of the pKD46 plasmid and grown at 30ºC in LB broth supplemented with 100 µg/mL ampicillin after electroporation.

Plasmids. Plasmid pKD46 is 6329 bp in size. It expresses the LRR system and contains the bla gene which encodes for ampicillin resistance. The plasmid pKD20 construct is identical to pKD46 (FIG. 1) except for the additional λ tL3 terminator in pKD46. This terminator makes pKD46 251 bp larger than pKD20 (10). Plasmid pACYC177 is 3941 bp in size and confers both kanamycin and ampicillin resistance. This plasmid was used as a template to amplify the kanamycin cassette with the KBP and LacKan primers.

Primer design. The KBP forward (KBP-F) primer (Table 1) is 77 bp in length, has a melting temperature of 69.9ºC and GC content of 56%. The first 39 bp of this primer is part of the lacI gene and was designed to align with the lacI start codon, GTG. The next 18 bp of the primer is a specialized region. This specialized region sequence was obtained from Amlani (2) and is complementary to the primer Pla46 which is part of the primer pair Pla46 and AA01 (2). This primer pair targets the 16S rRNA gene of anammox planctomycetes (2). The last 20 bp of the KBP primer sequence are the first 20 bp of the kanamycin gene of the pACYC177 plasmid and is the same sequence previously used by Broekhuizen et al (6). The KBP reverse (KBP-R) primer is 77 bp in length, has melting temperature of 71.8ºC and GC content of 48%. The first 39 bp of this primer are part of the lacI gene and were designed such that the sequence would include the stop codon AGT. The next 18 bp is the specialized region complementary to AA01 and the last 20 bp of the primer are the last 20 bp of the kanamycin gene which is also the same sequence previously used by Broekhuizen et al (6). The LacKan primers designed by Broekhuizen et al (6) were used alongside the KBP primers as controls in PCR and transformation experiments.

Plasmid isolation. Plasmids, pKD46 and pACYC177, were isolated using the alkaline lysis method (4). MG1655 and DH5α cells were grown overnight then 2 mL was centrifuged at 13,400 x g for 5 min at room temperature. Supernatant was discarded. The pellet was resuspended in 200 µL TE: RNase (10mM Tris-Cl, 1mM EDTA, 0.5 µg/µL ribonuclease A) and the tubes were vortexed. Next, 200 µL containing 0.2 M NaOH, and 1% sodium dodecyl sulphate was mixed with 200 µL 4 M potassium acetate and was added to the suspended pellet before the tube was inverted 3-5x and centrifuged as

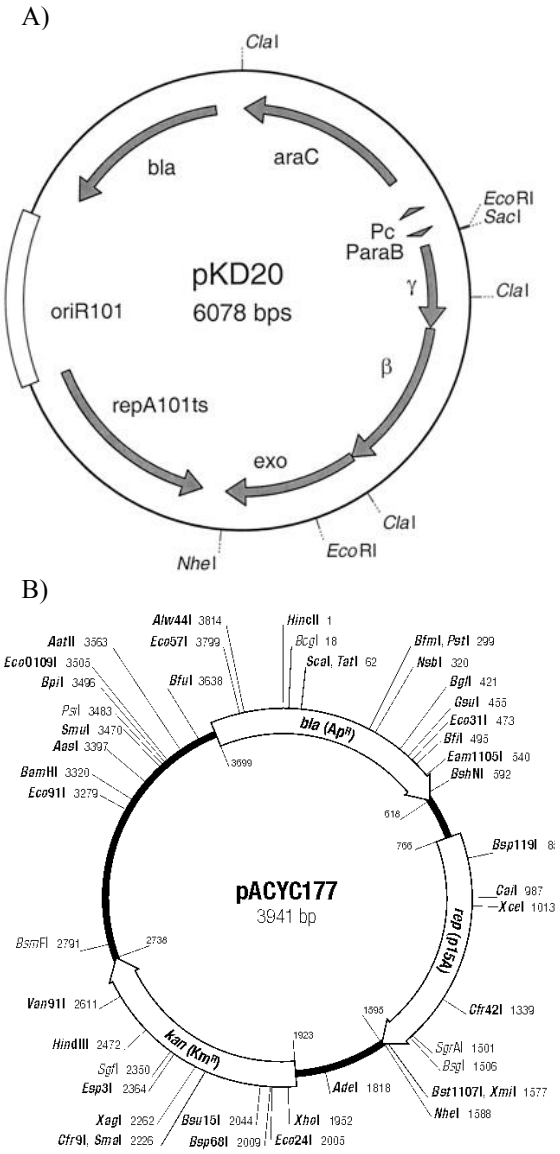

FIG. 1. A) Partial restriction digest map of plasmid pKD20. This construct is identical to pDK46 except for the addition of a λ tL3 terminator following the *exo* gene. B) Restriction digest map of plasmid pACYC177.

previously described. Supernatant was collected and added to tubes containing 600 µL chloroform and vortexed. Tubes were centrifuged at 13,400 x g for 1 min at room temperature. The upper aqueous layer was transferred to tubes containing 600 µL isopropanol, mixed and then centrifuged at 13,400 x g for 3 min at room temperature. Supernatant was carefully removed without disturbing the DNA pellet. Pellet was washed with 500 µL of 70% ethanol to remove salts and dried before it was resuspended in 50 µL TE buffer.

Table 1. The sequences and annealing temperatures of primer used in this study

Primer	Nucleotide Sequence (5' to 3')*	Annealing Temperature (^oC)	Reference
KBP-F	**TGAAACCAGTAACGTTATACGATGTCGC AGAGTATGCC**CGACTTGCATGCCTAATCCT TATGAGCCATATTCAACGG	65.5	This study
KBP-R	**GCGTATTGGGCGCCAGGGTGGTTTTTCT TTTCACCAGT**CTCGGGAACATATTCACCA ACTCATCGAGCATCAAATG	65.5	This study
LacKan-F	**AGGGTGGTGAATGTGAAACCAGTAACGT TATACGAT**TTATGAGCCATATTCAACGG	65.5	6
LacKan-R	**TCACTGCCCGCTTTCCAGTCGGGAAACC TGTCGTGC**AACTCATCGAGCATCAAATG	65.5	6
Pla46	GACTTGCATGCCTAATCC	55.9	1
AA01	CTCGGGAACATATTCACC	55.9	1

Genomic DNA extraction and purification. DNA was extracted using the Freeze/Thaw method outlined as follows. 2 mL of overnight culture was centrifuged at maximum speed for 1 min. The supernatant was discarded before 100 µl of TE buffer was added. The sample was frozen at -80°C for 5 min, and then thawed at 85°C for 1 min. This step was repeated for a total of 5 times, however, on the last thaw the sample was heated at 85°C for 5 min to reduce endogenous DNase activities. The sample was then microcentrifuged at maximum speed for 5 min. The resulting supernatant containing the genomic DNA was stored at -20°C until required.

Polymerase chain reaction amplifications. 2 µL of diluted DNA (1, 1/10, 1/100) was added to each 50 µL PCR reaction containing 1X PCR buffer, 0.25 mM dNTP, 2 mM $MgCl_2$, 0.2 µg/mL primers, 2.5 U Taq polymerase (Fermentas, Burlington, ON). PCR reactions were set up on ice and reactions were initially denatured for 4 min at 95°C. This denaturation was followed by 36 cycles of 1 min at 95°C, 1 min at the annealing temperature of the primer (Table 1), and 2 min at 72°C. Lastly, a final extension of 10 min at 72°C was done before the reaction was cooled to 4°C. A temperature gradient was initially used for all primers to determine the optimal annealing temperature. Negative controls to which no template DNA was added were included in every set of reactions. 5 µL of PCR reaction was added to 1 µL of 6x loading dye and run on a 1% agarose gel in 1x TAE Buffer (40 mM Tris pH 8.0, 1.14 ml glacial acetic acid, 1 mM EDTA), along with the Fermentas 1Kb MassRuler, GeneRuler (Cat #: SM0311). The gel was run at 105V for 45 min to 60 min then stained for 45 min in 0.2 mM ethidium bromide. The PCR product was visualized and photographed using UV illumination in an AlphaImager (Alpha Innotech Corporation).

Preparation of electrocompetent cells. 2 mL of C29 cells grown overnight in LB broth at 30°C was prepared. 1.25 mL of overnight culture was added to 125 mL of 1 mM L-arabinose LB broth and the culture was incubated with shaking at 30°C until the OD_{600} reached approximately 0.5-0.7 at that point the flask was chilled on ice for 20 min. All subsequent steps were done with equipment and reagents prechilled at 4°C. The culture was centrifuged at 4000 x g for 15 min and the supernatant was discarded. The pellet was successively washed with 125 mL, then 62.5 mL, and then 5 mL of chilled 10% glycerol by centrifuging at 4000 x g for 15 min between the washes. The final pellet was resuspended in 1.25 mL 10% glycerol and 100 µL aliquots were made in 1.5 mL microcentrifuge tubes. These microcentrifuge tubes were immediately frozen in a dry ice/ethanol bath and stored at -80°C (5).

Transformation. An aliquot of prepared C29 electrocompetent cells was thawed on ice. In a microcentrifuge tube, 40 µL of electrocompetent cells were mixed with 1-2 µL of DNA and incubated on ice for 1 min. The cells were then transferred to a chilled 0.2 cm BioRad electroporation cuvette and pulsed once with the BioRad MicroPulser using the program "Ec2" (5). Immediately 1 mL of 1 mM L-arabinose LB broth was added to the cuvette and the suspension was then transferred to a test tube and incubated at 30°C for 1 hour with shaking at 250 rpm. 10 µL and 100 µL of the transformation mixture were then spread on LB agar (LBA) containing the appropriate antibiotic selection: either 50 µg/mL kanamycin or 50 µg/mL ampicillin, or both. The transformations were also spread on LBA containing no selection to ensure that after the electroporation the cells were still viable. For the control transformations sterile water was electroporated instead of DNA. Transformations were first done to insert the pKD46 plasmid into the C29 cells and then these transformed cells were electroporated with the linear PCR construct.

Enzyme plate assay. Of the kanamycin resistant transformants obtained from the KBP PCR construct transformation, 70 colonies were randomly chosen. These colonies were grown overnight in 50 µg/mL kanamycin LB broth at 30°C. These cultures were then streaked onto LBA plates containing 50 µg/mL kanamycin, 100 µg/mL ampicillin, 20 mg/mL IPTG, and 50 mM 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) dissolved in DMSO (9) and incubated overnight to distinguish the presence of repressed or constitutive β-gal expression

RESULTS

The pACYC177 plasmid was isolated from DH5α cells at a final concentration of 72 µg/ml. The identity of the plasmid was confirmed through antibiotic susceptibility testing and restriction digests (data not shown). Subsequent PCR amplification using this plasmid as a template and the primers KBP-F/KBP-R or LacKan-F/LacKan-R yielded the respective expected bands at ~920 bp or ~880 bp (FIG. 2). The PCR reactions performed with annealing temperatures below 65.5°C (lanes 1-6, 11-16) gave a robust band for both primer sets, while those above this temperature (lanes 7-8, 17-18) produced weak or no bands compared with the controls (lanes 9-10, 19-20). Subsequently a number

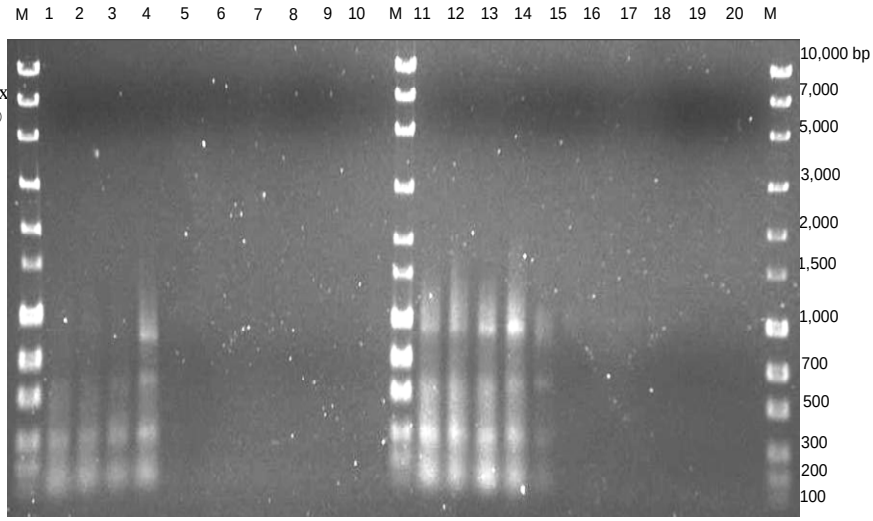

FIG. 3. 1% agarose gel electrophoresis of gradient PCR amplification products used for colony genotyping (5 µl sample + 1 µl 6x loading buffer). Lanes 1-10: 1/100 dilution of linear KBP PCR construct as template, lanes 11-20: 1/1000 dilution of the same DNA. PCR temperature gradient increasing from 51.8°C in lanes 1 and 11 to 72°C in lanes 10 and 20. Lane 4 and 14 show the expected ~850 bp band at 55.9°C among a series of putatively concatomeric PCR products.

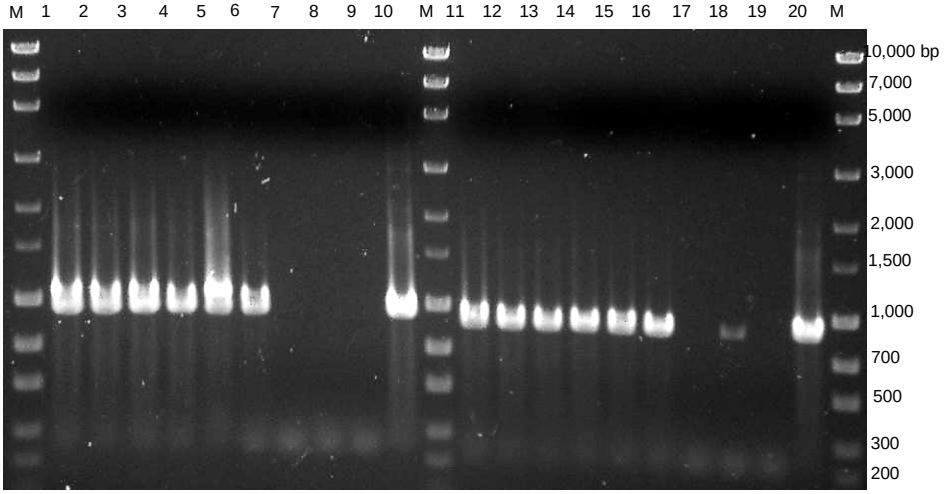

FIG. 2. 1% agarose gel electrophoresis of gradient PCR amplification products (5 µl sample + 1 µl 6x loading buffer) containing the kanamycin gene flanked by the novel linking regions and the *lacI* homologus regions (lanes 1-10) or kanamycin gene flanked by the lacI homologus regions of previous studies (lanes 11-20). 4 µl of DNA Mass ladder in lanes denoted M. Lanes 1-10 and 11-20 had an annealing temperatures of 52, 52.4, 56, 58.4, 63.2, 65.5, 70.1, 71.5, 52, 60.8°C, respectively. Negative and positive controls for the KBP and Lac-Kan primers are in lanes 9 and 19, 10 and 20 respectively. Subsequent amplifications at 65.5°C were used for transformation.

of PCR reactions were carried out at 65.5°C to produce the linear product used in later transformations.

Concentrated PCR products from both primer sets, the positive pACYC177 plasmid control and the negative water control were transformed into C29 cells harbouring the pKD46 plasmid. As seen in Table 2, kanamycin resistance was observed weakly in the positive control, since the pACYC177 plasmid contains a kanamycin resistance gene, and at a much higher rate in the KBP concentrated PCR product. This is in contrast to the variable but present growth of all samples on a non-selective LBA plates.

While a discrepancy was observed in the viability of colonies exposed to the linear insertion construct compared to the control electroporation cells, this is likely due to toxic remnants of the ethanol precipitation procedure used to concentrate the constructs before electroporation.

Following electroporation, results obtained from an examination of over 70 isolated colonies from water samples, pACYC177, and the kanamycin resistant KBP revealed well regulated expression of β-gal in all colonies examined with the enzyme plate assay.

Using a subset of the selected kanamycin resistant colonies and including the original linear KBP PCR construct as a positive control, PCR based genotyping with the primer set Pla46 and AA01 revealed that the PCR method was able to amplify a DNA band from the linear KBP PCR construct (FIG. 3) but was unable to amplify the corresponding band from the kanamycin resistant colonies assessed (data not shown). Figure 3 shows that incubation of the linear KBP PCR product with the Pla46 and AA01 primers, specific to the linking regions of the KBP insertion construct, produces an ~850 bp band at temperatures at and below 55.9°C (lane 4 and 14).

Table 2. Results of Transformation of *E. coli* C29 with various constructs

Sample	LB agar*		LB agar + Kanamycin	
	10ul	100ul	10ul	100ul
Water	~5000	~40000	0	0
pACYC177	2990	~11000	0	6
LacKan PCR product	140	2070	0	0
KBP PCR product	100	1280	25	334

*Values were corrected for a 1/10 dilution.

DISCUSSION

This study builds upon previous studies that look at the ability of the LRR system to cause site-specific mutagenesis of the lacI gene in E. coli C29 cells. Upon critical review of previous unsuccessful studies, two factors were initially identified that may have resulted in the failure of this site specific mutagenesis system: temperature, and L-arabinose concentration.

The pKD46 plasmid which contains the LRR system has a temperature sensitive origin of replication and will only replicate at 30°C (13). Previous unsuccessful studies have been culturing, at least at some point in time, the C29/pKD46 cells at 37°C (1, 6, 7, 10, 12, 14) which should have at least lead to partial curing of the pKD46 plasmid from the C29 cells. In this study, the cells were cultured exclusively at 30°C in accordance with previous successful studies (3, 8, 11).

The second factor that might have inhibited the success of the LRR system was the concentration of L-arabinose used. The recombinase genes are under an L-arabinose inducible promoter, therefore L-arabinose must be present in order for the system to function properly and cause site-specific mutagenesis. Previous, unsuccessful studies have either not used L-arabinose (6, 7, 12, 14) or have not specified the concentration used (1). A final concentration of 1 mM L-arabinose was used in accordance with previous successful studies (8, 11).

After the C29/pKD46 cells were electroporated with the PCR product, the constitutive expression of β-gal was tested by plating the transformants on media containing kanamycin that had been spread with X-gal. If lacI had been successfully disrupted then dark blue colonies would have arisen. If the transformation was not site-specific, indicating the LRR system did not work, then the colonies would be light blue or white. In this study, as seen in previous studies, although kanamycin colonies did arise, they were light blue or white. Since the KBP primers contain a specialized region (2), the kanamycin resistant colonies in the end product could be screened for random mutagenesis as opposed to transformation with the LacKan PCR product. As seen in Figure 3, amplification of the specialized region was successful off of the purified PCR product, but no amplification was obtained from the kanamycin resistant colonies (data not shown). The development of this band was most robust when using the 1/1000 dilution of the purified PCR product as a template. While the band of interest was present within a series of concatomeric PCR amplicons, the presence of products suggests that this method should be able to identify the presence of the construct within genomic DNA samples if the corresponding insert was present in the cells.

The initial concentration of cells for electroporation was approximately 10^9, and taking into account that survival rates are 20 to 80% (5), there should be at least 10^8 cells present after electroporation. The number of kanamycin resistant colonies that arose after electroporation with the KBP insert was approximately 1 in 10^5 (Table 2). Since random mutations are expected in 1 in 10^6 cells it is possible that the colonies that arose were due to random mutation alone. This suggests that temperature and L-arabinose concentration alone are not sufficient to cause the LRR system to be fully functional.

Near the end of the study another feature was identified that may have also contributed in the failure of the LRR system. The feature that was not addressed in this study is the presence of the restriction modification system in the C29 cells. Successful studies in the past that utilized the LRR system have the restriction modification system knocked out or mutated in the host (3, 8, 11). This might have prevented successful site-specific mutagenesis of the C29 cells even though the other factors were expected to work.

FUTURE EXPERIMENTS

In future studies, a strain with the restriction modification system knocked out or mutated should be used in accordance with previous successful studies (3, 8, 11). In this way, the effects of the C29 host strain can be examined as a possible reason for why the LRR system has been ineffective thus far.

An experimental approach utilizing the MG1655 strain, which lacks a restriction modification system, could be taken. Given the goal of targeted disruption of the lacI gene from the bacterial chromosome using a linear PCR insertion construct, this approach would examine the effects of the host strain restriction modification system under conditions which closely replicate this study.

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