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Attempts to Construct an Enlarged pUC19 via Insertion of HindIII-digested Coliphage λ DNA

Muhamed Amirie, Isabelle Cheng, Joanne Cho, Viet Vu
Department Microbiology & Immunology, University of British Columbia

The plasmids pUC19 and pBR322 are commonly used as vectors in molecular biology contexts. It has been observed that when *Escherichia coli* cells are co-transformed with these two plasmids, pUC19 is preferentially retained while pBR322 is excluded. Several hypotheses have been proposed to account for this phenomenon, one of which is the larger size of pBR322. The objective of this study was to construct an enlarged pUC19 vector (pVACC13W) via insertion of λ DNA fragments in order to test the role of size on the exclusion effect observed when pBR322 was co-transformed with pUC19 into *E. coli* DH5α. A HindIII restriction digest was performed on the λ genome, and the resulting fragments subjected to agarose gel electrophoresis. Two fragments, sizes 2322 bp and 2027 bp, were extracted from the gel and a ligation reaction performed at the HindIII restriction locus within the multiple cloning site of pUC19 in an attempt to produce plasmids of sizes 5008 bp and 4713 bp respectively. However, no colonies were obtained when these ligation reactions were transformed into *E. coli* DH5α, potentially due to a failure to inactivate T4 ligase in the ligation mixture prior to transformation or contamination of the DNA samples resulting in inefficient ligation. The results of this study indicate that isolation of λ DNA fragments via agarose gel extraction introduces the potential issues of low DNA yield and contaminated samples, suggesting that an alternate method – perhaps the amplification of desired fragments using PCR – may be more effective.

When *E. coli* cells are co-transformed with the plasmids pUC19 and pBR322, the pUC19 is preferentially retained in the resulting transformants (1). A number of mechanisms have been proposed to account for this phenomenon, including the larger size of pBR322, and the presence of the *rop* gene in pBR322 which negatively regulates copy number (2). However, preliminary evidence suggests that the *rop* gene is not the causative factor in pUC19's preferential selection in co-transformation with pBR322 (1), leading us to investigate the alternative explanation.

Previous work has shown that cells transformed with larger plasmids exhibit a longer lag period (3), and that transformation efficiency decreases with plasmid size (4); both of these observations suggest that the size difference between pUC19 and pBR322 may be the cause of the preferential selection of pUC19 following co-transformation into *E. coli* DH5α. In order to examine the effect of the greater size of pBR322 – 4361 bp in comparison to pUC19's 2686 bp – we attempted to construct an enlarged recombinant pUC19. With this we aimed to investigate the role of plasmid size on the observed exclusion effect of pBR322 upon co-transformation with pUC19.

A prior experiment attempted to clone enlarged pUC19 vectors by inserting fragments of λ DNA amplified by PCR and digested with BamHI, but was not successful in constructing the desired plasmid (5). This study attempted a different approach, by performing HindIII restriction enzyme digests of λ DNA and ligating the resulting fragments with the pUC19 vector. Additionally, the λ DNA fragments used in this study do not overlap with those used in the attempt by Chow (5). pUC19 possesses a multiple cloning site (MCS) containing multiple restriction endonuclease-specific loci (2), including a single digestion site for HindIII which cleaves double-stranded DNA at the sequence 5'-A↓AGCTT-3' (6). Upon HindIII cleavage, the enzyme creates overhangs in digested DNA fragments, which can then be ligated with complementary overhangs of other fragments or digested vectors. Furthermore, the λ genome contains six HindIII restriction sites, producing eight DNA fragments of various sizes upon digestion (7). In preparation to study the effect of plasmid size on preferential selection in co-transformation, we aimed to ligate HindIII-digested λ fragments of sizes 2322 bp and 2027 bp with pUC19 to produce 5008 bp and 4713 bp plasmids respectively, designated pVACC13W. However the desired clones were not isolated, and further research is required both in production of an enlarged pUC19 construct using this method and in the investigation of the effect of this vector on the rate of pUC19 retention when co-transformed with pBR322.

MATERIALS AND METHODS

Bacterial strains and culture methods. *E. coli* DH5α cells, including cells containing pUC19, were provided from the MICB 421 culture collection in the Department of Microbiology and Immunology at the University of British Columbia. Cells were grown overnight in Luria-Bertani broth (LB) at 37°C at 200 RPM in a rotary shaker for use the following day. LB broth was prepared by adding 10.0 g/L tryptone, 10.0 g/L NaCl, 5.0 g/L yeast extract to deionized water. LB agar was prepared by adding 1.5% agar to LB broth prior to autoclaving.

Restriction digest of λ DNA. A restriction digest of λ DNA with HindIII (Invitrogen, Cat. 15207-020), was performed in a 40 µl reaction with 10 µg λ DNA, 1X Buffer R (Thermo Scientific, #BR5), sterile H_2O, and 5 units/µg DNA of HindIII. λ DNA was digested at 37°C for 1 hour and stored at -20°C upon completion.

Gel extraction of HindIII-digested λ DNA fragments. λ DNA restriction endonuclease digests were run on a 1.5% agarose (Bio-Rad, 161-3101) gel in 1X Tris-acetate-EDTA (TAE) Buffer. The gel was run at 97 volts until distinct separation of the 2322 bp and 2027 bp fragments was apparent. HindIII/λ DNA fragments (Invitrogen, Cat 15612-013) were run as a control. Portions of the

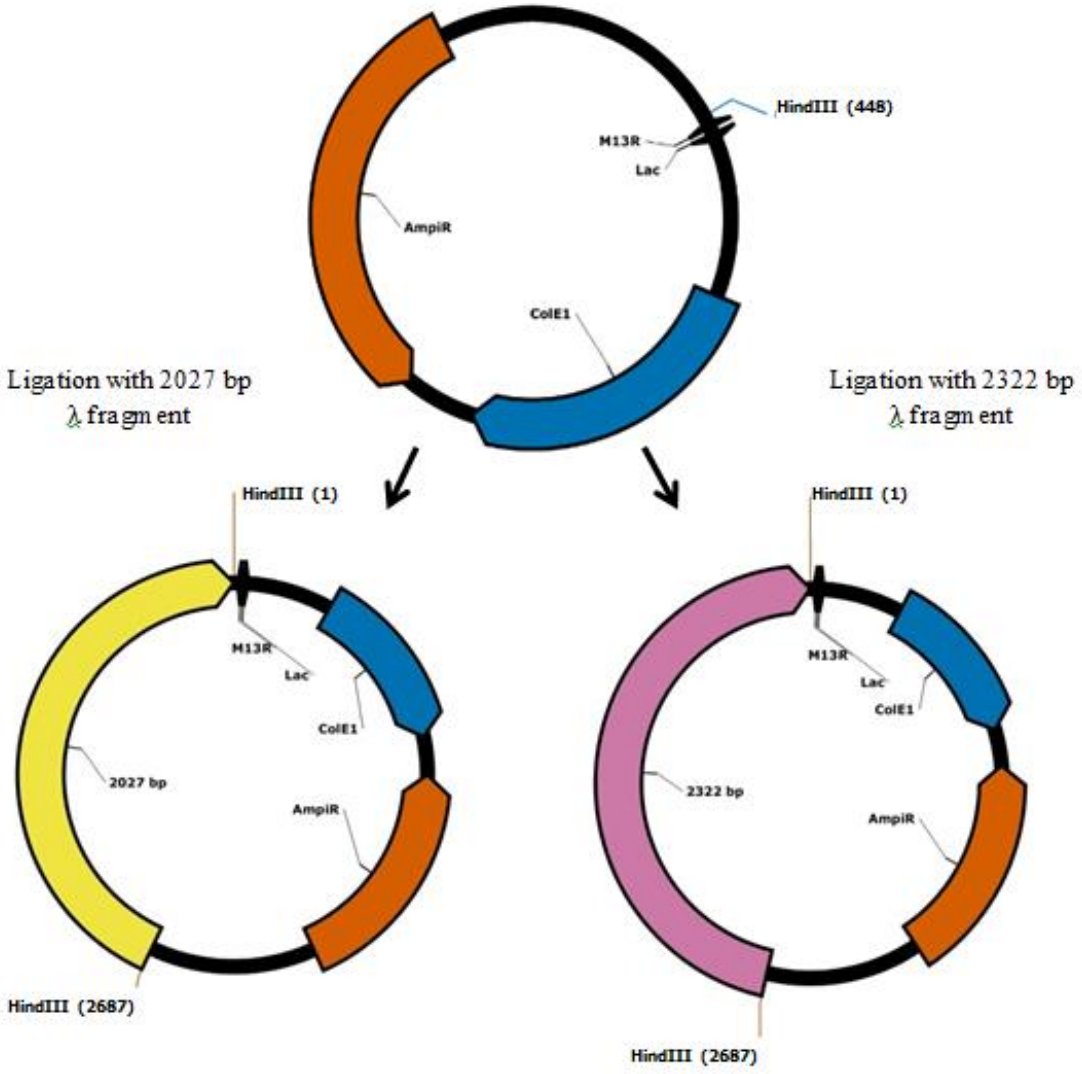

FIG. 1. Wild-type pUC19 and intended novel pVACC13W plasmids. 2027 bp and 2322 bp fragments ligated into the pUC19 MCS via the HindIII restriction sequence would produce 4713 bp and 5008 bp plasmids respectively. Insertion into the MCS disrupts the *lacZα* gene. The ampicillin-resistance gene (*ampiR*) and *ori* site (ColE1) are not affected by the insert.

gel containing the desired fragments visualized under UV light were excised using a sterile scalpel. Isolated λ DNA fragments were purified and extracted from the gel using the GenElute Gel Extraction Kit (Sigma-Aldrich, NA1111) according to the manufacturer's protocol (8). Extracted λ DNA was centrifuged at 16 000 x g for 2 minutes and transferred to a new tube after extraction to remove residual silica from the extraction process.

Purification of λ DNA fragments. Isolated λ DNA fragments were concentrated via ethanol precipitation. 1 volume of λ DNA, 1/10 volumes 3M sodium acetate, and 2 volumes cold 100% ethanol were combined and placed on ice for 30 minutes. The mixture was spun at 20 000 x g for 15 minutes, decanted, and 500 µl 70% ethanol was added before spinning again at 20 000 x g for 10 seconds. The solution was decanted and the resulting pellet allowed to air dry for 5-7 minutes, followed by resuspension in 25 µl H_2O.

Isolation and digestion of pUC19. pUC19 was isolated from *E. coli* DH5α cultures using the GeneJET Plasmid Midiprep Kit (Thermo Scientific, K0481) according to the manufacturer's specifications (9). The plasmid was digested with HindIII at 37°C for 1 hour in a solution containing 592 ng pUC19, 1X Buffer R (Thermo Scientific, #BR5), sterile H_2O, and 5 units/µg DNA of HindIII. pUC19 digests were dephosphorylated with 5 units of Antarctic Phosphatase (New England Biolabs, M0289S) and 10X Antarctic Phosphatase Buffer (New England Biolabs, B0289S) at 37°C for 15 minutes, and the phosphatase was deactivated at 70°C for 5 minutes.

Ligation of pUC19 with λ DNA fragments. Ligation of 150 ng digested pUC19 with 450 ng λ DNA was performed in a 42 µl reaction with 1 unit T4 DNA ligase (Fermentas, EL0018), 1X T4 DNA ligase Buffer (Fermentas, B69), and was incubated at 22°C for 10 minutes.

Preparation of electrocompetent cells. *E. coli* DH5α cells were made electrocompetent according to the MicroPulsar Electroporation Apparatus Operating Instructions (Bio-Rad) protocol: High Efficiency Electrotransformation of *E. coli* (10).

LB broth was substituted for all media used in the protocol. Electrocompetent cells were stored in 10% glycerol at -80°C.

Electroporation. Electroporation was performed according the MicroPulsar Electroporation Apparatus Operating Instructions (Bio-Rad) protocol: High Efficiency Electrotransformation of *E. coli* (10). Transformants were plated on LB with ampicillin (50 µg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) to select for colonies containing intact plasmids and distinguish colonies containing the desired constructs, respectively.

RESULTS

HindIII-digestion of λ DNA. Restriction enzyme digests of λ DNA by HindIII were expected to generate bands of 23130, 9416, 6557, 4361, 2322, 2027, 564, and 125 bp respectively (7), the largest six of which were observed on the gel (Fig. 2). The uppermost band in Lanes 4 and 5 represents the largest two of these fragments, which due to the relatively high agarose concentration of the gel (1.5%) did not entirely separate during the run. The 2322 bp and 2027 bp bands were clearly visibile and beginning to separate, and we chose to isolate both of these bands in preparation for incorporation into pUC19 and construction of pVACC13W.

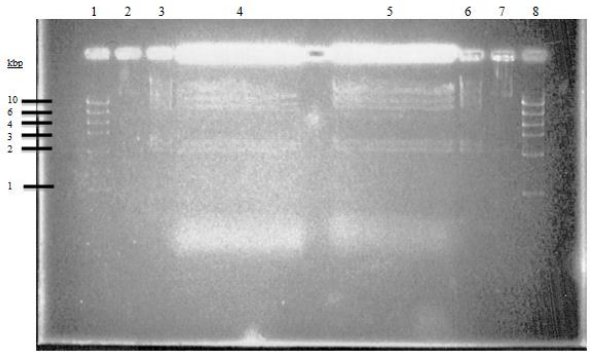

FIG. 2. HindIII restriction digest of λ DNA. Lanes 1 and 8: Invitrogen High DNA Mass Ladder. Lanes 2 and 7: undigested λ DNA. Lanes 3 and 6: Invitrogen HindIII/λ DNA fragments. Lane 4: HindIII-digested λ DNA (10 µg). Lane 5: HindIII-digested λ DNA (5 µg).

Isolation of λ DNA fragments. The absorbance profile for our λ DNA fragments after gel extraction and ethanol purification did not display an apparent peak, even though the absorbance increased slightly at approximately 244 nm (Fig. 3). As the highest point on the graph was at 220 nm (the lowest displayed wavelength), it appeared that the profile peaked prior to 220 nm. Although 10 µg of λ DNA was digested (Fig. 2), the two extracted fragments together accounted for only 0.896 µg, or 8.96% of the total DNA. Following extraction and ethanol preciptitaion of these fragments, 25 µl of DNA at a concentration of 22.6 ng/µl was isolated, for a total of 0.565 µg of insert DNA. As the manufacturer claims DNA recovery of up to 80% (8) our recovery of 63% was considered quite reasonable. However, the absorbance profile for the isolated DNA did not exhibit a peak at 260 nm, nor did the 260/230 ratio fall in the expected range of 1.8-2.2 (Fig. 3). A low 260/230 ratio generally indicates contamination with organic compounds or various salts, and can also indicate the presence of residual components from agarose gels (11). We extracted fragments from a TAE-based gel, so EDTA and agarose are both likely contaminants of our sample. While the concentration of DNA was indicated to be 22.6 ng/µl, the purity of the sample remained unconfirmed. Therfore, the measured concentration of 22.6 ng/µl and the estimated recovery of 63% are likely significant overestimates, which may have negatively impacted the subsequent ligation reaction.

Isolation of pUC19 from *E. coli* DH5α. The absorbance profile for the isolated pUC19 exhibited a weak peak from approximately 255-258 nm, which gradually decreased after this point (Fig. 4) The sample displayed a 260/280 ratio of 2.01 which was higher than the expected 1.8, indicating the potential presence of RNA contamination (12). The 260/230 ratio of 2.03, however, was within the expected range and suggested that significant levels of protein contaminants were not present. While the indicated DNA concentration was 14.8 ng/ul, this is likely an overestimate due to the presence of contaminants in the sample.

Transformtion of pUC19 and pVACC13W into *E. coli* DH5α. Following ligation, the resulting DNA was transformed into *E. coli* DH5α cells via electroporation and transformants plated on media allowing for selection of colonies containing the pVACC13W vector, making the use of the plasmid's *lacZα* and ampicillin resistance genes (2). After electroporation, however, there were no resulting transformants able to grow on LB + ampicillin (Table 1), indicating that pVACC13W was not present in the cells – if it was present, we expected that the ampicillin resistance gene within the plasmid would facilitate growth on an ampicillin-containing medium. However, transformants from the second transformation able to grow on LB without ampicillin were observed (data not shown), indicating that the cells were not killed by the electroporation process. In addition, cells transformed with pUC19 were able to grow in the presence of ampicillin (Table 1), indicating that pUC19 was taken up by the cells. These transformants, however, exhibited a lower than expected transformation efficiency of 8.9×10^2 cfu/µg – transformation of pUC plasmids into *E. coli* DH5α cells via electroporation can achieve transformation efficiencies of $10^9 – 10^{10}$ cfu/µg (13).

DISCUSSION

Previous experiments have attempted to enlarge pUC19 via the insertion of specific λ DNA sequences amplified by PCR (5). This study aimed to construct an enlarged recombinant pUC19 by performing a HindIII restriction digest of λ DNA, and ligating the resulting 2322 bp and 2027 bp fragments into the HindIII restriction site within pUC19, in order to produce novel pUC19 constructs (pVACC13W) of 5008 bp and 4713 bp (Fig. 1). Though the PCR amplified λ DNA fragments used in the Chow study did not encode any full length λ genes and were therefore unlikely to have been a factor

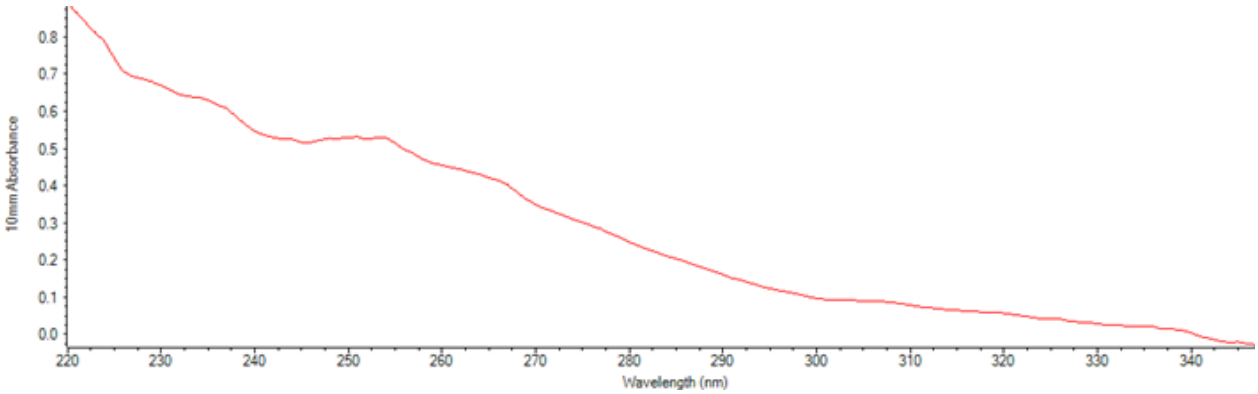

FIG. 3. Absorbance profile for gel-extracted 2027 bp and 2322 bp λ DNA fragments.

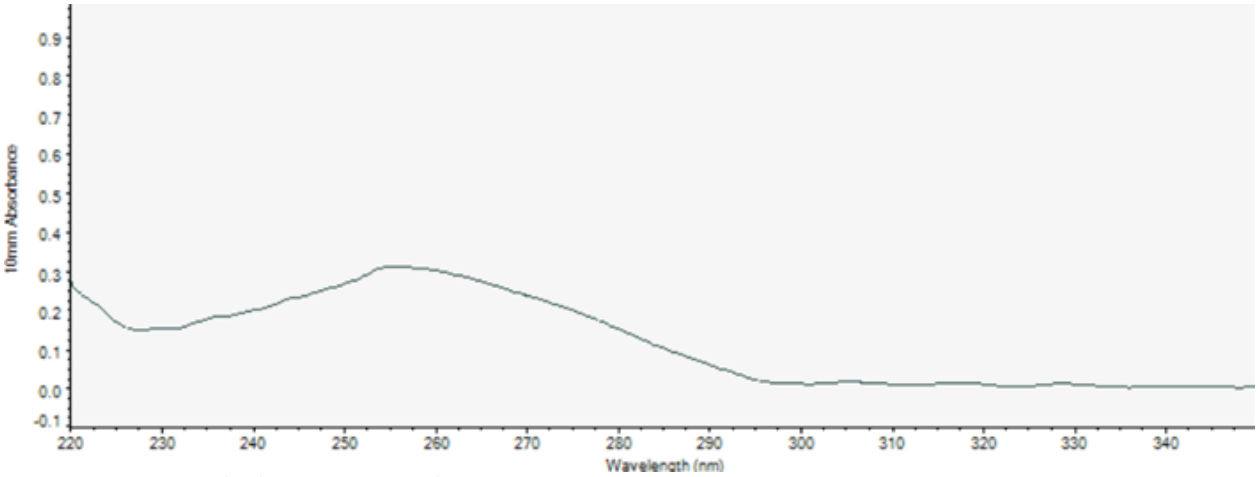

FIG. 4. Absorbance profile for pUC19 isolated from *E. coli* DH5α cells.

in the lack of success of their experiments via production of λ gene products, we chose to use fragments from different regions of the λ genome in order to eliminate this potential source of error. The 2322 bp fragment of the λ genome, from position 25158 – 27479 (7), contains the predicted protein-coding domain *ea31* of unknown function (14). The 2027 bp product, from position 23130 – 25157 in the λ genome (7), contains only a portion of the protein-coding domain *ea47* which is also of unknown function (14). While limited information has been gathered on these genes, they have been shown to be produced early in the infection cycle (15), and both *ea31* and *ea47* have been implicated in conferring resistance to infection via a mechanism involving the LamB receptor (16). While it was counted upon that neither of the potential coding regions within these insert fragments would impact the fitness or activities of *E. coli* DH5α host cells upon transformation, the possibility remains that at high concentrations these gene products may interfere with the native porin activity of LamB, perhaps inhibiting sugar transport in the host cells (17).

Following transformation of ligation reactions into *E. coli* DH5α cells via electroporation and plating of the resulting transformants on LB containing ampicillin and X-gal, no colony growth was observed (Table 1), indicating that these cells did not contain pVACC13W. The same transformants, however, were capable of growth on LB alone (data not shown), and therefore were not killed by the electroporation procedure. Furthermore, cells electroporated with pUC19 displayed growth on LB containing ampicillin (Table 1), suggesting that the cells used were indeed electrocompetent, and were taking up plasmids through the transformation process. These results appear to indicate that the issue occurred at the level of the ligation reaction as opposed to the transformation procedure. Due to the low amount of pUC19 DNA isolated and digested, we were unable to run the digested plasmid on a gel in order to determine if compete digestion had occurred. Furthermore, the likely EDTA contamination of our DNA samples may have negatively impacted ligation efficiency, as could have the above optimal concentration of DNA used in our ligation reaction (14.3 µg/ml as opposed to the ideal 1-10 µg/ml) (18). Any of these factors may have contributed to a lack of success at the ligation step.

However, a second possibility remains, that failing to inactivate T4 ligase – which is susceptible to heat inactivation at 65°C for 10 minutes (19) – before

TABLE 1. Transformations of pUC19 and pVACC13W into *E. coli* DH5α plated on LB + ampicillin. Cells transformed with pUC19 were able to grow on LB + ampicillin, whereas cells transformed with pVACC13W were not. Two transformations were attempted with pVACC13W, using 1.5 µl and 10 µl of ligation mix respectively.

Plasmid	Amount of DNA Transformed (µg)	Number of Colonies (cfu/ml)	Transformation Efficiency (cfu/µg)
pUC19	0.750	6.4×10^2	8.9×10^2
pVACC13W	0.143	0	N/A
pVACC13W	0.021	0	N/A

transformation may have severely impacted transformation efficiency. It has been shown that leaving T4 ligase actve can produce up to a 550-fold decrease in transformation efficiency (20), and this may have contributed to the lack of successful pVACC13W transformants. This is also supported by the low transformation efficiency observed in the pUC19 transformants (Table 1), and as more plasmid DNA was used in this transformation than in those with ligation reactions, is it plausible that a combination of T4 ligase remaining active and insufficient concentrations of pVACC13W was responsible for the lack of transformants.

This study aimed to construct an enlarged pUC19 vector via the insertion of HindIII-digested λ DNA fragments into the HindIII restrction site of the pUC19 MCS. However, a lack of confirmation of the purity of isolated pUC19 and λ DNA prevented conclusive determination of the efficacy of these methods in constructing novel pUC19 vectors, and a suboptimal ligation protocol may have inhibited the isolation of the desired plasmids. Initial DNA isolation and ligation experiments have provided a basis for future troubleshooting in the construction of enlarged pUC19 vectors using this technique.

FUTURE DIRECTIONS

Future research should build off of the troubleshooting done in this study in order to construct an enlarged pUC19 vector. Two of the largest challenges faced in this study were the low concentration and presence of contaminants in the isolated HindIII digested λ DNA. These issues may be resolved by performing a number of HindIII restriction digests of the λ genome, performing separate gel extractions and ethanol purifications for each, followed by pooling all aliquots and further purifying this stock. If these methods are sufficient to isolate more highly concentrated λ DNA of greater purity, the subsequent ligation of the λ fragments to pUC19 may be successful. However, the issue of insufficient DNA yields may be avoided altogether by designing primers and performing PCR to amplify the λ genome fragments of interest and performing a HindIII restriction digest from this point. By digesting only the fragments we intend to ligate with pUC19 as opposed to digesting the entire λ genome and isolating the desired bands, we should be able to isolate larger quantities of insert DNA. Furthermore, isolating and ligating larger quantities of DNA will allow us to run the ligation products on a gel to determine whether the reaction produces plasmids of the intended size. Based on the challenges encountered in this study, the PCR approach appears to be the most promising in resolving these issues.

Additionally, experiments should be performed to test the effect of inactivating T4 ligase before proceeding with transformation. Two ligation reactions could be set up in parallel, one in which T4 ligase has been inactivated following the ligation reaction, and one in which it has not. Transforming and plating *E. coli* DH5α cells with the resulting ligated products, would determine whether or not the inactivation of T4 ligase indeed affects transformation efficiency.

Once an enlarged pUC19 vector has been produced and isolated, the next step is to use this plasmid to investigate the exclusion effect of pBR322 when co-transformed with pUC19 into *E. coli* DH5α. This can be accomplished by performing a series of co-transformations: one with pUC19 and pBR322, one with pUC19 and the enlarged pUC19 construct, and one with pBR322 and the enlarged pUC19 construct. By utilizing the ampicillin and tetracycline resistance genes present in pUC19 and pBR322 respectively, selective plating following transformation will allow the identification of colonies that received each plasmid. From these experiments, we expect that pUC19 will be preferentially selected over both pBR322 and the enlarged pUC19 construct, and that pBR322 and the enlarged pUC19 construct will be retained in *E. coli* DH5α transformants at similar rates. If these results are observed, it will indicate that size is the dominant factor in the exclusion of pBR322 when co-transformed with pUC19 into *E. coli* DH5α.

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