Journal of Experimental Microbiology and Immunology (JEMI) Vol. 8:40-46

Attempt to Construct a *Rop*$^+$ pUC19 by Using *Nde*I, *Aat*II, and *Afe*I Restriction Endonucleases with Separate Blunt and Staggered-end Ligations

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Cloning vector, pUC19, was originally derived from parent plasmid pBR322. But pUC19 has a higher copy number in host cells compared to pBR322. pBR322 plasmids possess a functional *rop* gene, expressed by readthrough transcription of the upstream *tet* gene, which generally controls copy number by facilitating the association of RNA II and RNA I molecules. It is theorized that the lack of a functional *rop* gene in pUC19 and the presence of a point mutation in the coding region of RNA II is responsible for the increase in copy number. In this study, it was attempted to create a *rop*$^+$ pUC19 plasmid construct to investigate the effect of a functional *rop* gene on pUC19 copy number compared to the unmodified pUC19 and the parent pBR322 plasmid. The first attempt to ligate the pBR322 fragment encoding the *tet* and *rop* genes into the pUC19 plasmid in place of the existing partial *rop* gene resulted in no clones. Experimental evidence showed that an inability to ligate *Nde*I cut DNA ends was a likely problem. An alternate method was explored that involved first excising the *tet* coding region from pBR322 with a different restriction enzyme and recircularizing the intermediate *tet*$^-$ pBR322 plasmid. Clones were successfully obtained that contained the correct intermediate plasmid. Subsequently, the *rop* gene along with the upstream *tet* gene P2 promoter and -10 and -35 regions could be excised and ligated into the pUC19 plasmid as before. Due to time constraints, this final step will need to be performed in future experiments which can then determine whether the lack of a *rop* gene in pUC19 is the sole reason for its high copy number compared to pBR322.

Both pBR322 and pUC19 plasmids are cloning vectors derived from ColE1-type replicons (7). Although pUC19 was originally derived from pBR322, it has been observed that pUC19 has a higher copy number in host cells compared to pBR322 (7). This phenotypic difference has been attributed pUC19's lack of a functional *rop* gene and the presence of a point mutation in the coding region of RNAII (6). During normal DNA replication in pBR322, a precursor primer RNA transcript (RNA II) is first synthesized by host RNA polymerase which then hybridizes to a DNA sequence near the origin of the plasmid. However, the bound RNA II precursor must be cleaved by RNase H to become an active primer for DNA synthesis to occur (7). In order to control the rate of plasmid replication, an RNA I transcript which is antisense to RNA II is also transcribed. Interaction of RNA I with precursor RNA II transcripts causes a conformational change in RNA II that inhibits its binding to the template DNA (7). Since RNase H only cleaves bound RNA II, RNA II primer formation is inhibited and so, thus, is plasmid DNA replication.

In addition, the rop protein also plays a role in control of plasmid copy number in the host cell. This 63 amino acid protein, in pBR322 replication, functions to promote interaction between RNA I and RNA II transcripts to allow more extensive hybridization between the molecules such that DNA replication is further inhibited by hindering activation of the RNA II primer (7). Therefore, plasmid copy number is negatively regulated by the presence and expression of a functional *rop* gene through an anti-sense control mechanism (6).

In the case of pUC19 plasmids, the point mutation in the coding region of RNA II inhibits RNA I and RNA II interactions and blocks RNA I control on DNA replication (7). Furthermore, the lack of a functional *rop* gene decreases RNA I control on DNA replication since its binding to RNA II is no longer aided. As a result, the copy number of pUC19 is significantly greater than pBR322 in cells. While high copy number allows efficient "preparation of plasmid DNA and high-level expression of proteins", it frequently causes physiological stress and toxicity to cell growth (2). Thus, understanding control of copy number in cloning vectors is very important.

This study focused on investigating whether the lack of a functional *rop* gene in pUC19 is solely responsible for its high copy number. To conduct this experiment, an attempt was made to produce a *rop*$^+$ pUC19 plasmid for comparison to pBR322 copy number. pUC19 possesses a partial *rop* gene in its

sequence but lacks the ribosome binding site and the first 157bp of the coding region. There is no apparent promoter directly upstream of the *rop* gene ribosome binding site (RBS). It has been determined from previous studies that transcription into the *rop* gene region occurs by readthrough transcription from the *tet* gene (4). As such, it was necessary to move the *tet* gene P2 promoter region as well as the -10 and -35 regions along with the *rop* gene to produce a functional gene in the pUC19 plasmid construct. Since the only variable that should have been present for the test was the introduction of a functional *rop* gene into pUC19, the *tet* gene coding region which was carried along needed to be excised before experimentation. Thus, the resulting pUC19 plasmid construct was to possess a complete *rop* gene along with its RBS, *tet* gene P2 promoter, and -10 and -35 regions in place of the existing partial *rop* gene. By comparing the copy number of unaltered pUC19, *rop*$^+$ pUC19 plasmids, and pBR322 plasmids, it can be determined what effect a functional *rop* gene has on plasmid copy numbers in the host cell and whether the rop protein is the major form of plasmid copy number control lacking in the pUC19 plasmids.

MATERIALS AND METHODS

Plasmid isolation. Using the Qiagen QIAprep® Spin Miniprep Kit (Cat.# 27104), plasmids were isolated following the protocol for low-copy plasmids from the QIAprep® Miniprep Handbook. Culture tubes containing 10 mL of Luria Bertani (LB) broth with ampicillin (100µg/mL) were prepared and inoculated with *Escherichia coli* DH5α. The *E. coli* DH5α strain grown overnight possessed either the pUC19 or the pBR322 plasmid. A significant change that was made to the protocol was to allow the elution buffer to sit in the column for an extra minute and subsequently spin for 2 minutes rather than the recommended 1 minute to maximize the volume of eluate collected.

pBR322 plasmid DNA. Commercially available pBR322 plasmid DNA isolated from *E. coli* (*dam*$^+$, *dcm*$^+$) was purchased from Fermentas Life Sciences (Cat.# SD0041) in a 0.5 µg/µL concentrated stock solution.

Phenol/chloroform purification and ethanol precipitation of DNA. To purify and concentrate the DNA, Invitrogen phenol:chloroform (Cat.# 15593-031) was added at 1:1 volume to the dilute DNA sample and the two layers vortexed vigorously together in a microcentrifuge tube. The mixture was spun briefly in a tabletop microcentrifuge to separate the two layers and the top aqueous layer was carefully pipetted off without touching the liquid interface and saved in a new tube. The volume of the recovered DNA sample was measured. The salt concentration was then adjusted by adding 1/10 volume of 3 M sodium acetate at pH 5.2 and thoroughly mixing. One volume of ice cold 100% isopropanol was then added and, again, mixed well. The tube was placed at -20°C at least 1 hour to allow the DNA to precipitate. The sample was then spun at maximum speed in a tabletop microcentrifuge at 4°C for 15 minutes. After carefully decanting the supernatant, 1 mL of 70% ethanol was added and mixed. The sample was again spun at maximum speed in the microcentrifuge at 4°C for 10 minutes and the supernatant carefully pipetted off and discarded. The tube with the remaining DNA pellet was then placed in the Eppendorf Vacufuge™ and dried at 30°C for approximately 20 minutes until the pellet was transparent. The pellet could then be resuspended in TE buffer or pH 7.0 distilled H_2O.

Restriction endonucleases digest conditions. All plasmid digests were performed with the following restriction endonucleases: *Nde*I (NEBiolabs Inc., Cat.# R0111S), *Aat*II (NEBiolabs Inc., Cat.# R0111S), *Pst*I (Invitrogen Corp., Cat.# 15215-015), *Nde*I (Fermentas Life Sciences, Cat.# ER0501), *Afe*I (NEBiolabs Inc., Cat.# V0213S). Plasmid pBR322 digests with *Nde*I (NEBiolabs Inc.), *Aat*II, and *Pst*I: 2 µg DNA, 20 U *Nde*I enzyme, 20 U *Aat*II enzyme, 10 U *Pst*I enzyme, 1X NEBuffer 4. Plasmid pBR322 digests with *Afe*I: 2 µg DNA, 10 U *Afe*I enzyme, 1X SEBuffer Y. Plasmid pUC19 digests with *Nde*I (NEBiolabs Inc.) and *Aat*II: 2 µg DNA, 20 U *Nde*I enzyme, 20 U *Aat*II enzyme, 1X NEBuffer 4. Plasmid pBR322 digests with *Nde*I (NEBiolabs Inc.) only: 2 µg DNA, 20 U *Nde*I enzyme, 1X NEBuffer 4. Plasmid pBR322 digests with *Nde*I (Fermentas Life Sciences) only: 2 µg DNA, 20 U *Nde*I enzyme, 1X Buffer R. All reactions were made up to a final volume of 20 µl with distilled H_2O. The digest reactions were incubated at 37°C for 2 hours or overnight (approx. 17 hours). Thereafter, the restriction endonucleases were inactivated at 75°C for 20 minutes.

Agarase digest of agarose gel bands. Desired DNA bands were cut out of 0.8% low melting point agarose (Invitrogen, Cat.# 15517-022) and melted at 70°C for 15 minutes. The tube was then equilibrated to 42°C for 5 minutes before addition of agarase (Fermentas Life Sciences, Cat.# E00461) at 1 U/100 mg of gel. The digest reaction was allowed to proceed at 42°C for 1 hour. At the completion of the digest, 1/10 volume of 3M sodium acetate (pH 5.2) was added to the tube and mixed well. The tube was then chilled on ice for 5 minutes. Subsequent centrifugation at 15,000 x g pelleted the undigested carbohydrates. The supernatant was carefully pipetted off the loose pellet and transferred to a clean tube. The DNA sample was then subjected to phenol/chloroform purification and ethanol precipitation as described above for the ligation reactions.

Ligation reactions. Blunt-end ligations of *Afe*I cut pBR322 fragments: 200 ng purified pBR322 fragment (2866bp), 1 U T4 DNA Ligase (Invitrogen Corp., Cat.# 15224-017), 1X DNA Ligase Reaction Buffer (Invitrogen Corp., Cat.# 46300-018). Staggered-end ligations of *Nde*I, *Aat*II, and *Pst*I cut pBR322 inserts with *Nde*I and *Aat*II cut pUC19 vectors: 90 ng of purified pBR322 insert (2366bp), 30 ng of purified pUC19 vector (2434bp), 0.1 U T4 DNA Ligase, 1X DNA Ligase Reaction Buffer. Staggered-end ligations of *Nde*I cut pBR322 plasmids: 150 ng of purified cut pBR322 (4361bp), 0.1 U T4 DNA Ligase, 1X DNA Ligase Reaction Buffer. All reactions were made up to a final volume of 20 µL with distilled H_2O. The ligation reactions were allowed to proceed at 14°C (blunt-end) or 16°C (staggered-end) overnight for approximately 17 hours. Five-to-ten replicate ligation reactions were pooled and ethanol precipitated as described above to concentrate the recircularized DNA and remove salts from the ligation reaction before bacterial transformation by electroporation.

Transformation of cells by electroporation. Transformation of the electro-competent cells (UBC Teaching Lab, frozen stock) was performed using the electroporation protocol for *E. coli* that is described in the Operating Instructions Manual Section 5.1 for the Bio-Rad MicroPulsar™ Electroporation Apparatus (Cat.# 165-2100). Briefly, 40 µL aliquots of electrically competent cells stored at -80°C were thawed on ice. Concentrated DNA resulting from pooled ligation reactions were added to each tube of thawed cells in 50-150 ng amounts in less than 5 µL volumes. The cells and DNA were mixed gently in the tube and then transferred down one side of an ice-cold 0.2 cm GenePulser Cuvette (Bio-Rad, Cat.# 165-2086) and allowed to sit on ice for approximately 1 minute. After electroporation of the cells on program EC2 in the MicroPulsar™, 1 mL of LB broth was immediately added to the cuvette to resuspend the cells. The cell suspension was transferred to a sterile microcentrifuge tube and shaken horizontally at 37°C for 1.5 hours to allow recovery. Two hundred microlitres of the culture was plated on an LB plate with ampicillin (100 µg/mL) and incubated face-down at 37°C overnight.

Screening of mutant clones. Ligated *Afe*I cut pBR322 fragments were screened by testing for loss of tetracycline resistance. Colonies resulting from the plated cells transformed with *Afe*I cut pBR322 ligation product were individually picked up with a sterile toothpick and dropped into 1 mL of LB broth with ampicillin (100 µg/mL) and grown shaking in a glass test tube at 37°C for approximately 2 hours. The broth cultures were then spotted on replicate plates with one LB plate containing ampicillin (100 µg/mL) and a second LB plate containing ampicillin (100 µg/mL) and tetracycline (25 µg/mL).

RESULTS

Ligation of pBR322 fragments encoding *tet* and *rop* genes into pUC19 vectors. The first digest involved the enzymatic cleavage of pBR322 plasmids with *Nde*I, *Aat*II, and *Pst*I which resulted in three distinct fragment sizes of approximately 2600bp, 1300bp, and 600bp. The purpose of *Pst*I in this digest was to cleave the unwanted 1995bp fragment resulting from *Nde*I and *Aat*II digestion to allow a cleaner separation of the similarly sized 2366bp desired fragment. Of the resulting fragments, the 2600bp fragment was predicted to possess the *tet* and *rop* genes according to the pBR322 restriction map (Figure 1). The second digest involved the enzymatic cleavage of pUC19 plasmids with *Nde*I and *Aat*II which resulted in two distinct fragments sized at approximately 2600bp and 300bp. The 2600bp fragment, predicted to be 2434bp in size according to the pUC19 restriction map (Figure 2), was expected to encode the majority of the pUC19 plasmid sequence with the exception of the partial *rop* gene open reading frame (ORF) that was excised in the digestion.

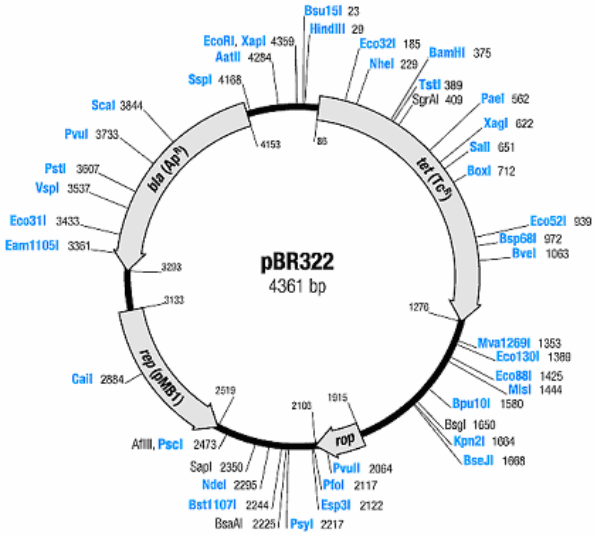

FIG. 1 Restriction map of the pBR322 plasmid. Digestion of this plasmid with restriction endonucleases *Nde*I, *Aat*II, and *Pst*I at positions 2296, 4284, and 3607 respectively should result in fragments approximately 2400bp, 1300bp, and 700bp in size. The largest fragment was predicted to possess the portion of the pBR322 plasmid encoding the *tet* and *rop* genes.

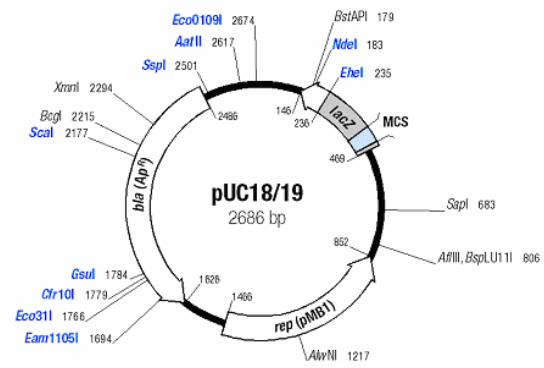

FIG. 2 Restriction map of the pUC19 plasmid. Digestion of this plasmid with restriction endonucleases *Nde*I and *Aat*II at positions 183 and 2617 respectively should result in fragments approximately 2400bp and 250bp in size. The 2400bp fragment should encode the pUC19 plasmid sequence with the exception of the partial *rop* gene ORF.

Transformation of electrically competent *E. coli* DH5α cells with 50ng or 150ng of pooled and purified DNA from the ligation reactions produced no colony growth on LB plates in the presence of ampicillin. While the transformation control with 100ng of uncut pBR322 resulted in the growth of 196 colonies, repeated efforts with increased transformation recovery time with the vector and insert ligation product still resulted in a lack of viable clones capable of growing on LB medium with ampicillin. An *Nde*I cut pBR322 ligation reaction conducted in parallel also resulted in no colony growth on LB medium with ampicillin.

The ligation products remaining after the bacterial transformations were run on an agarose gel (Figure 3). The vector and insert ligation product showed a bright band approximately 4800bp in size in addition to a fainter 2400bp fragment band (Figure 3, lane 2). Only a 4361bp fragment band was observed in the *Nde*I cut pBR322 ligation product (Figure 3, lane 3). It was observed that a new 4800bp band was produced in the vector and insert ligation reaction. No new bands were produced in the *Nde*I cut ligation reaction.

Excision of the *tet* gene and recircularization of pBR322. Commercially available pBR322 plasmids digested with *Afe*I resulted in fragments approximately 3000bp, 800bp, and 300bp in size. Ligation and recircularization of the largest (3000bp) fragment was assumed successful because transformation of *E. coli* DH5α cells by electroporation with about 200 ng of ligation product DNA resulted in the growth of 36 clones which potentially had the intermediate *tet*$^-$ pBR322 plasmid of interest. Remaining ligation product was observed to contain DNA fragments sized at approximately 2800bp, 5600bp, and 6600bp. New 6600bp and 5600bp bands were produced due to the ligation reaction in addition to the 2800bp band corresponding to the original fragments.

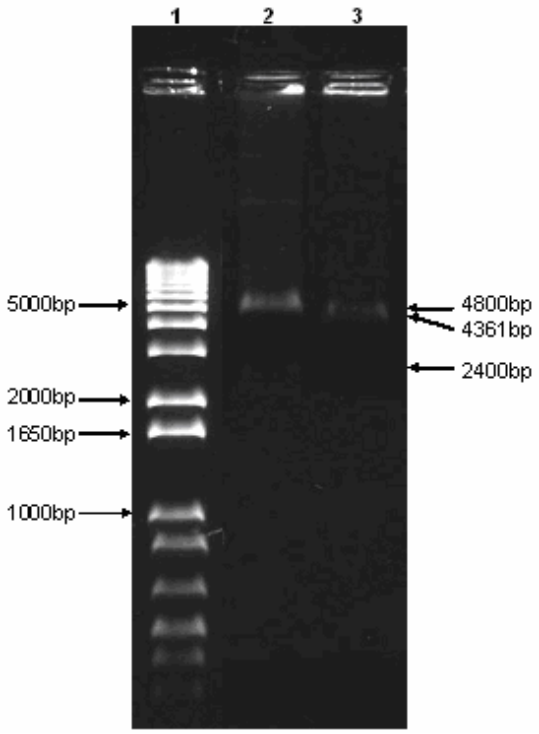

FIG. 3 Analysis of ligation reaction products in a 0.8% agarose gel. The ligation product of the pBR322 insert (2366bp) and pUC19 vector (2434bp) showed a clear 4800bp fragment and a fainter 2400bp band (lane 2). The ligation product of *Nde*I cut pBR322 only showed a single fragment approximately 4400bp in size (lane 3). A new 4800bp band was produced in the vector plus insert ligation reaction whereas no new bands were produced in the *Nde*I cut vector ligation reaction. Lane 1 is the 1kb Plus DNA Ladder.

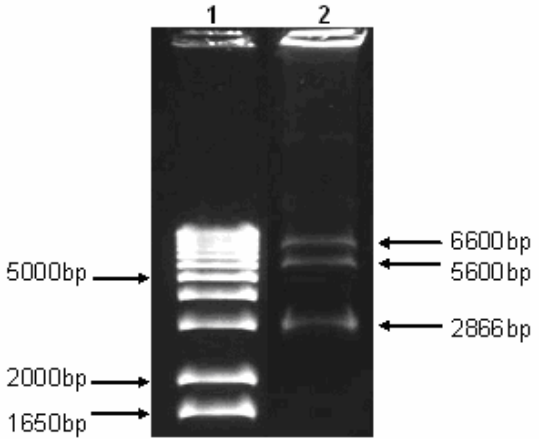

FIG. 4 Analysis of ligation products in a 0.8% agarose. Ligation of the *Afe*I cut pBR322 (2866bp) fragment (lane 2) showed new 6600bp and 5600bp bands in addition to the 2800bp band corresponding to the original fragment. Lane 1 was the 1kb Plus DNA Ladder.

Thirty of the 36 clones were screened for a loss in tetracycline resistance by duplicate spot-plating on two different types of media: LB with ampicillin, LB with both ampicillin and tetracycline. It was observed that all 30 (IN051 → IN0530) of the clones were capable of propagating in the presence of ampicillin but not in the presence of tetracycline.

DISCUSSION

Attempts to construct *rop*$^+$ pUC19 plasmids were conducted to investigate the effect of a functional *rop* gene on the copy-number of pUC19 plasmids in *E. coli* DH5α bacterial cells. Attempts were made to ligate fragments later referred to as "insert DNA", encoding the complete *tet* and *rop* genes excised from pBR322 plasmids, into pUC19 plasmid vectors in which the existing partial *rop* gene had been cut out to construct the final *rop*$^+$ pUC19 plasmid. The lack of colonies obtained through this method was suspected to be due to inadequate transformation recovery time, DNA purity, insufficient DNA in the transformation, or not enough ligation time. But further attempts to produce a clone possessing this intermediate plasmid with increased bacterial transformation recovery time of 3 hours, greater purification of ligation DNA, increased amount of DNA used in the transformation, and extended ligation time of 48 hours still resulted in no viable clones. Thus, it is unlikely that the cloning failed due to these suspected problems.

It was then tested whether it was a problem with cell viability and DNA uptake which resulted in the observation of no clones. Since transformation of the bacteria with uncut pBR322 plasmids resulted in the growth of 196 colonies, it was evident that the transformed cells were viable and DNA uptake was adequately efficient that about 200 clones could be expected from the plating of each transformation reaction. Therefore, transformed cell viability and DNA uptake were not the issue in the lack of clones obtained.

The observation of a new 4800bp fragment after the ligation reaction of the vector and insert (Figure 3, lane 2) suggested that the ligation was successful in producing vectors ligated to insert sequences. However, it was unclear whether the ligated product had completely recircularized to produce a viable plasmid or remained a nonviable linear DNA molecule. Thus, it was suspected that a linear DNA ligation product was being taken up by the cells resulting in clones that were unable to propagate in the presence of ampicillin because of lack of expression of a functional beta-lactamase (*bla*) gene that is encoded on the vector segment of the ligation product (Figure 2). Ligation and recircularization of the vector and insert is required for expression of the *bla* gene that confers ampicillin resistance.

An issue that has been presented before is an uncharacterized difficulty in ligation of *Nde*I cut DNA ends. The lack of *Nde*I cut end ligation of the vector and insert fragments could have resulted in no recircularization and produced a nonviable linear DNA product. To test this possibility, pBR322 plasmids cut once with *Nde*I were ligated in the same way as the pUC19 vector and pBR322 insert ligation reaction and used to transform electrically competent cells identical to those used previously. No colonies resulted from the transformation, thus providing evidence that the *Nde*I cut ends were not ligating back together under the ligation conditions. It was then reasonable to suspect that the inability to produce viable clones might have been due to difficulty in ligation of the pBR322 fragment with the pUC19 vector. The ligation product from the *Nde*I cut pBR322 ligation reaction was run on an agarose gel (Figure 3, lane 3) showed that, indeed, the *Nde*I cut pBR322 fragment ends were not ligating back together since recircularization would have led to the production of a bulkier relaxed open-circle DNA form which should have migrated at a slower rate compared to the original linear fragment. In contrast, it was apparent from sizing of the ligation product from the pBR322 insert and pUC19 vector ligation reaction (Figure 3, lane 2) that there was successful ligation of two 2400bp original fragments to form larger DNA molecules of approximately 4800bp.

There are many possible products of a vector and insert ligation reaction. One reason that may explain why transformation of cells with the insert and vector ligation product did not produce viable clones is that complete recircularization was not achieved. In this case, the ligation of one end of the insert fragment to one end of the vector fragment can produce a linear 4800bp DNA molecule as seen in figure 3 but the lack of recircularization would lead to a nonviable product which could not provide ampicillin resistance to the host cell. It is plausible, taking into account the evidenced inability to ligate *Nde*I cut ends, that the vector and insert fragments were only able to ligate together by their *Aat*II cut ends to produce a linear 4800bp DNA molecule. Similarly, ligation of *Aat*II cut ends of vector fragments to each other will still produce nonviable 4800bp linear DNA molecules for the same reasons as above.

A second reason why the ligation product DNA may have been nonviable is that indiscriminate ligation lead to recircularization of two insert fragments with each other. Since the insert fragment from pBR322 encoded neither the origin of replication nor the *rep* region needed to promote replication, a circularized DNA molecule consisting of two ligated insert fragments would be unable to replicate in the cell. In

addition, this plasmid product would not impart ampicillin resistance to the host cell since it does not encode the *bla* gene needed to produce beta-lactamase so the clone would not have survived on the selective media in any case. However, a 4800bp DNA molecule can still be produced in this scenario leading to a nonviable plasmid product.

Given that an intermediate plasmid could not be produced through the first method, an alternative method was explored to produce the same final *rop*$^+$ pUC19 plasmid construct. The *tet* gene from the pBR322 plasmid was to be excised before the *rop* gene was cut out to be ligated into the same pUC19 vector as before. An intermediate *tet*$^-$ pBR322 plasmid was produced by first excising the *tet* gene from pBR322 plasmids by restriction digest with *Afe*I. Recircularization of the *tet*$^-$ pBR322 DNA fragments by ligation and transformation of *E. coli* with the ligation product resulted in 36 ampicillin-resistant clones which may or may not have contained the desired plasmid. Through a screen for a loss of tetracycline resistance, it was determined that all 30 of the clones possessed plasmids encoding the *bla* gene but not the *tet* gene. Further screening is still required to ensure that the plasmids found in the clones are true *tet*$^-$ pBR322 plasmids and not pUC19 contamination which has the same antibiotic resistance phenotype. Due to time constraints, the alternative method was only explored until this point. However, frozen stocks of all of the screened clones were saved for future experimentation.

FUTURE EXPERIMENTS

There are a few possible directions that future experiments can take to explore questions that arose in the process of conducting this study. One direction includes a continuation of the current study by completing the intermediate *tet*$^-$ pBR322 plasmid screening. Further screening of the clones will involve isolation of plasmids from expanded cultures of the frozen stocks by minipreps. Some of the plasmids can then be digested with *Pvu*II and run on an agarose gel to ascertain the identity of the plasmids. pUC19 plasmids, which exhibit the same antibiotic resistance phenotype as the intermediate *tet*$^-$ pBR322 plasmids, are cut in two places by *Pvu*II and the digest product should contain equal numbers of 322bp and a 2364 bp fragments. The *tet*$^-$ pBR322 plasmids, on the other hand, possess only one *Pvu*II restriction site per plasmid so the digest product would be expected to consist of linear 2866bp fragments. Clones possessing plasmids identified by the digestion fragmentation pattern as being the correct intermediate construct can then be propagated to isolate plasmids for the second part of the *rop*$^+$ pUC19 construction process.

To produce the final *rop*$^+$ pUC19 construct, the intermediate *tet*$^-$ pBR322 plasmids should first be mutated at the *Nde*I cut site to create a new and unique cut site not currently present on the intermediate plasmid (eg. *Nhe*I, *Bam*HI, *Tst*I, *Sgr*AI, *Pae*I, *Xag*I, *Sa*II, *Box*I, *Eco*521, *Bsp*681, *Bve*I, *Mva*1269I, *Eco*130I, *Eco*881, *Mls*I, *Bpu*10I, *Bsg*I, *Kpn*2I, *Bse*JI) to avoid issues with *Nde*I cut end ligation. It is important to choose a restriction site that would also be unique on the pUC19 plasmid since the *Nde*I cut site on pUC19 must also be similarly mutated. A possible method of mutation may be site-directed mutagenesis. Digestion of the intermediate plamid with *Aat*II and the restriction enzyme that cuts at the newly created cut site will then excise fragments encoding the complete *rop* gene along with the upstream *tet* gene promoter and -10 and -35 regions. These fragments, henceforth referred to as the pBR322 *rop* inserts, can then be ligated into pUC19 vectors cut with the same enzymes to form the final *rop*$^+$ pUC19 constructs. To prevent vector self-ligation, treatment of the vectors with alkaline phosphatase will remove the 5' phosphates to promote subcloning of untreated inserts into the vectors. Transformation of *E. coli* DH5α cells with these final constructs resulting in viable colonies will complete the construction process to produce the clones possessing the *rop*$^+$ pUC19 constructs of interest for study. A comparison of plasmid copy-number in host cells between the *rop*$^+$ pUC19 and the unmodified pUC19 plasmids will answer the question of whether the lack of a functional *rop* gene in pUC19 is the sole reason why copy-numbers are elevated compared to pBR322 plasmids.

ACKNOWLEDGEMENTS

I would like to take the opportunity to thank Jennifer Sibley and Dr. William Ramey for all their time, counsel, technical assistance, and expertise without which this study could not have been conducted. My MICB 447 classmates were also of great assistance in this endeavor through helpful suggestions and continual support and encouragement throughout the semester.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 2002. Short Protocols in Molecular Biology. Fifth edition. John Wiley & Songs, Inc. USA.
2. **Cronan, J.** 2005. A family of arabinose-inducible *Escherichia coli* expression vectors having pBR322 copy control. Plasmid. in press.*
3. **Fang, D.** 2004. Attempts to use PCR site-directed mutagenesis to create a non-functional *rop* gene in

the plasmid pBR322. J. Exp. Microbiol. Immunol. **6:** 45-51.

4. **Li, T. K., Y. A. Panchenko, M. Drolet, and L. F. Liu.** 1997. Incompatibility of *Escherichia coli rho* mutants with plasmid is mediated by plasmid-specific transcription. J. Bacteriol. **179:** 5789-5794.
5. **Horwitz, M. S. Z.** 1989. Transcription regulation *in vitro* by an *E. coli* promoter containing a DNA cruciform in the '-35' region. Nucleic Acids Res. **17:** 5537-5545.
6. **Sozhamannan, S., and B. L. Stitt.** 1997. Effects on mRNA degradation by *Escherichia coli* transcription termination factor Rho and pBR322 copy number control protein Rop. J. Mol. Biol. **268:** 689-703.
7. **Sozhamannan, S., J. G. Morris, Jr., and B. L. Stitt.** 1999. Instability of pUC19 in *Escherichia coli* transcription termination factor mutant, *rho026*. Plasmid. **41:** 63-69.