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Attempts to Use PCR Site-Directed Mutagenesis to Create a Non-functional *rop* Gene in the Plasmid pBR322

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The co-transformation of two ColE1-type plasmids, pBR322 and pUC19 results in the exclusion of pBR322 from the cell. Several factors could potentially contribute to this exclusion effect; one potential factor is the Rop protein that is only encoded in the pBR322 plasmid. The Rop protein stabilizes the interaction between the replication primer, RNA II, and its antisense strand, RNA I. By complexing RNA I to RNA II, the replication of the plasmid is inhibited. Therefore, the absence of *rop* in pUC could directly lead to a higher replication rate and eventually a higher copy number, which then leads to the eventual exclusion of pBR322 during a co-transformation with pUC. This study attempted to create a pBR322 plasmid with a non-functional *rop* gene, by inserting stop codons into the *rop* gene using PCR site-directed mutagenesis. Theoretically, by creating a non-functional *rop* gene, the RNA II transcript should be free to anneal to the plasmid in order to act as a primer for replication. This increased rate of replication for pBR322 could potentially lead to a higher copy number, and perhaps result in the cancellation of the exclusion effect in a co-transformation experiment. However, the incorporation of the mutations into the plasmid was unsuccessful and so the co-transformation step was never completed.

ColE1-type plasmids, such as pBR322 and pUC19, rely on the synthesis of RNA II, a RNA primer precursor, in order to replicate. The RNA II primer hybridizes to its template DNA to become active and is cleaved at a specific site by RNase H. The cleavage results in a free 3'OH end that is accessible to the DNA polymerase for DNA synthesis. There is also a second RNA molecule, RNA I, that acts as an antisense strand, as it is transcribed to be complementary to the 5' end of the RNA II transcript. The resulting interaction between RNA I and RNA II inhibits the primers binding activity to the DNA template and subsequently halts the replication of the plasmid (3).

In addition to RNA II, there is a second molecule, Rop, which can also control the replication of ColE1-type plasmids. Rop is a 63 amino acid protein that prevents an increase in the plasmid copy number. Moreover, deleting the *rop* gene resulted in a twofold increase in copy number (3). Therefore, the actual function of Rop is to accelerate and stabilize the binding complex of RNA I and RNA II (3). Noticeably, the *rop* gene can be found in the pBR322 plasmid, but not in the pUC19 plasmid. This deletion of *rop* in pUC may result in its high copy number compared to that of its parental plasmid and to that of pBR322 (6). In fact, experiments involving the co-transformation of pUC19 and pBR322 resulted in approximately 90% of the cell culture carrying only the pUC19 plasmid, resulting in the exclusion of pBR322 from the host cells (6).

There are many possibilities that could answer some of the questions raised by the above co-transformation result. Possibilities include the role of Rop in the pBR322 plasmid. The pUC19 plasmid does not encode for a *rop* gene, which means there is no specific protein stabilizing the RNA I – RNA II complex. Without the complementation of RNA I to RNA II, the RNA II primer could hybridize to its DNA template and initiate replication. Therefore, the exclusion effect of pBR322 could be a direct result of the activity of Rop, which leads to a decreased replication rate, and eventually a lower copy number, and the exclusion of the plasmid from host cells.

A second possibility could be the presence of a point mutation in the RNA II transcript sequence of the pUC19 plasmid. There is a single G→A mutation located in nucleotide 112 of the RNA II transcript. This mutation could potentially alter the pUC19 copy number because it could affect the length and efficiency of the antisense RNA I. The RNA I transcript is shortened by 3 nucleotides from its 5'end and leads to a less effective binding of RNA I to RNA II, when compared to the binding of RNA I to RNA II in the pBR322 plasmid, which does not contain a point mutation in its RNA II transcript. Therefore, the decreased efficiency of RNA I to complement to RNA II in the pUC19 plasmid, could result in its higher copy number and thus lead to the exclusion effect of pUC19 on pBR322 (3).

A final possibility to explain the results seen in the co-transformation experiment is the size of each plasmid. The pBR322 plasmid (4361bp) is almost twice as large as the pUC19 (2686bp) plasmid and this increase in size could directly result in a lower replication rate of the pBR322 plasmid. Because of its size, pBR322 may take a longer time to be replicated

and since the cell population is continually dividing, this increased time used for replication could result in the elimination of pBR322 due to the competitive advantage of pUC19 (6).

This study focuses on the role of Rop in the pBR322 plasmid; specifically, whether a non-functional *rop* gene in pBR322 will eliminate the exclusion effect of pUC19 on pBR322. Therefore, the hypothesis is that if the *rop* gene in pBR322 was mutated and made non-functional, then the copy number of pBR322 would increase and result in a higher percentage of cells containing the pBR322 plasmid when co-transformed with pUC19. The experimental strategy used to create a non-functional *rop* gene without deleting the entire gene was to use PCR site-directed mutagenesis to create a stop codon within the first 6 codons of the *rop* transcript. Through the use of specifically designed primers, a second stop codon was also created in the 8th codon of the *rop* transcript, as well as a unique *Kpn*I enzyme restriction site within the primer region of the *rop* transcript. The exact locations of the restriction site and stop codons can be seen in figure 1.

The original experimental strategy was to use a single PCR step, where the entire plasmid was to be replicated in one PCR reaction, and then the newly replicated plasmid transformed into *E. coli* DH5α cells. However, due to difficulties with the PCR, the method was modified to contain a second set of primers, as well as a digestion and ligation step, prior to the transformation into cells.

MATERIALS AND METHODS

Plasmid isolation. Using the Qiagen QIAfilter Plasmid Maxi Kit (12263), the pBR322 plasmid was isolated from *E. coli* DH5α grown in 250mL LB broth (1) to an OD_{660} of approximately 0.7 when 0.28μg/mL of choramphenicol was added, and the culture allowed to grow overnight.

PCR reactions. Primer #1: 5'-CATGGCCCGGT ACCTCTGAAGCTAGACATTAA-3' (binds to base pairs 1944 – 1975 on pBR322), Primer #2: 5'-TAATGTCTAGCTTCCAGAGGTACCGGGCCATGT -3' (binds to base pairs 1944 – 1975 on pBR322), Primer #3: 5'- CGTGCTGCTAGCGCTATATGCGT TGA -3' (binds to base pairs 228 – 253 on pBR322), Primer #4: 5'-AGCGCTAGCAGCACGCCATAGTG ACT-3' (binds to base pairs 203 – 228 on pBR322). The primers were designed using the DNASTAR, Inc. Lasergene DNA and Protein Analysis software (Version 6, Wisconsin, USA) and made by the UBC Nucleic Acid Protein Service Unit (NAPS). Reaction Conditions (all chemicals were from the Invitrogen Corporation): 2U *Taq* DNA polymerase enzyme, 0.2mM dNTPs, 1X PCR Reaction Buffer (minus $MgCl_2$), 1.5mM $MgCl_2$, 2pmol/μL (for each) of primers 1, 2, 3, and 4, 100ng pBR322 template DNA (cut with the restriction endonuclease *Nhe*I) (7). The reaction was conducted using the Bio-Rad Gene-Cycler PCR machine with the following program: 1st cycle: 3 min at 94ºC, 2nd – 39th cycles: 1 min at 94ºC, 1 min at 48ºC, 8 min at 68ºC, 40th cycle: 60 min at 68ºC. Primers 1 and 4 were used in one reaction and primers 2 and 3 were used in a separate reaction.

Restriction endonuclease digest conditions. *Nhe*I (Invitrogen Corporation) digests: 180ng - 1μg DNA, 1U *Nhe*I enzyme, 1X REACT© 4 buffer. *Kpn*I (Invitrogen Corporation) digests: 180ng - 300ng DNA, 1U *Kpn*I enzyme, 1X REACT© 4 buffer. For both enzymes, incubate at 37ºC for 3 hours.

Ligation reactions. 100ng purified PCR product (2656bp fragment), 198ng purified PCR product (1753bp fragment), 1X Ligase Reaction Buffer (Invitrogen Corporation), 1U T4 DNA Ligase (Invitrogen Corporation). Total volume of reaction: 45μL using dH_2O.

Transformation of cells via electroporation. The competent cells were prepared by Jennifer Sibley using a protocol found in Current Protocols in Molecular Biology (1). The cells were then transformed with a standard electroporation protocol described in the user manual for the Bio-Rad MicroPulser™ electroporation apparatus (2). Pipette DNA in less than 5μl (different volumes between 1μL and 5μL of DNA can be used if the optimal amount is unknown) into one side of an ice-cold electroporation cuvette. Then pipette 50μL of competent plasmid free *E. coli* DH5α into the same cuvette (to the same side as the DNA). Wipe the cuvette dry and place into the Bio-Rad Micropulser Electroporation Device and pulse using program EC2 (2). After electroporating, pipette 500μL of soc broth (2) into the cuvette and pipette up and down gently. Transfer the solution of broth, cells, and DNA into a sterile culture tube and incubate the solution of cells at 37ºC for 1 hour with shaking. After the incubation, plate the cell solution onto LB plates with ampicillin (50μg/mL) (1) and incubate the plates (upside down) overnight at 37ºC. The next day, pick the colonies (36) and grow on LB plates with both ampicillin (50μg/mL) and tetracycline (12μg/mL). Then incubate the plates overnight at 37ºC. The next morning, pick 12 colonies and grow up in 3mL culture tubes with LB broth, ampicillin (50μg/mL), and tetracycline (12μg/mL). Miniprep each of the 12 cultures using GibcoBRL Life Technologies Concert™ Rapid Plasmid Miniprep System (#11453-024).

Restriction endonuclease digests to check for mutations in *rop*. Digest the minipreps with *Kpn*I using the above restriction digest conditions. Also run parallel *Nhe*I digestions for each miniprep to use as

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                                                  G ACC (T)    (T)
GTGACCAAACAGGAAAAAACCGCCCTTAACATGGCCCGCTTTATCAGAAGCCAGACATTAA
CGCTTCTGGAGAAACTCAACGAGCTGGACGCGGATGAACAGGCAGACATCTGTGAATCGCT
TCACGACCACGCTGATGAGCTTTACCGCAGCTGCCTCGCGCGTTTCGGTGATGACGGTGAA
AACCTCTGA
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FIG. 1 Sequence of the *rop* gene and the mutations to be incorporated into the sequence via site-directed mutagenesis PCR. The sequence is listed from 5' to 3' and shows the ATG start codon boxed in a rectangle. The unique *Kpn*I site incorporated into the gene is shown with an underline and the bases above the sequence show the nucleotides that are to be incorporated in order to create the restriction site. The stop codons to be incorporated to create a non-functional *rop* gene are also shown and the bases to be incorporated are indicated by the bracketed nucleotide.

controls, as well as lambda (λ) DNA to use as a positive control.

RESULTS

Complete PCR site-directed mutagenesis. The pRB322 plasmid was isolated from *E. coli* DH5α and used unmodified as the template in a PCR reaction. The initial plan was to use primers 1 and 2, as shown in figure 2a, in the same site-directed mutagenic PCR reaction in order to generate a complete, double-stranded plasmid that contains mutations encoding stop codons within the first few codons of the *rop* gene. The primers were designed to create stop codons at positions 1960 and 1966, as well as a unique *Kpn*I restriction site at position 1952, which can be seen in figure 2a. However, multiple attempts of the PCR reaction resulted in no product production; an indication that the primers were not hybridizing to the template or elongating in the reaction in sufficient quantity.

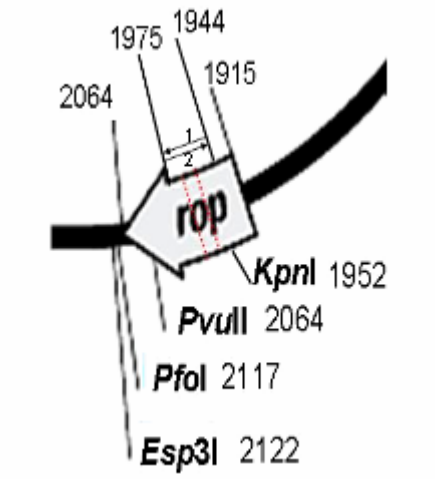

FIG. 2a The *rop* gene segment of the pBR322 plasmid. The arrows show the direction and hybridization position of primers 1 and 2. The location of the incorporated *Kpn*I site is also shown in the figure, as well as the location of each of the two stop codons, which is indicated by the dashed lines on the *rop* gene.

Partial PCR site-directed mutagenesis. Due to the inability to use the initial two primers to fully replicate new DNA with mutations in the *rop* gene, a second set of primers were designed, as shown in figure 2b. This second set of primers (primers 3 and 4) is 26 base pairs long and is located at position 228 on the template, each headed in opposite directions. These two primers were then coupled with the first set of primers and the pBR322 template, which was linearized with a *Nhe*I digestion. The result was the production of two DNA fragments that were 1753bp and 2656bp in size. Figure 3 shows three lanes for each fragment and also appears to show extra bands on the gel with sizes that are insignificant. Lanes 1, 2, and 3 have additional faint bands that are about 7000bp, 1100bp, and 550bp in size. Lanes 5, 6, and 7 also have faint bands that are about 3500bp and 700bp in size. This indicates that extra PCR products are being produced in the PCR reaction, in addition to the expected DNA fragments. After about 30 – 32 cycles, a certain threshold is passed and more regions of the plasmid can be amplified, producing the faint bands (W. Ramey, personal communication).

Digestions and ligations. The PCR-produced DNA fragments were digested for three hours using the restriction enzymes *Kpn*I and *Nhe*I. *Kpn*I should remove 19 base pairs from the end of the fragment that is 1753bp in size and 13 base pairs from the end of the fragment that is 2656bp in size. *Nhe*I should remove 7 base pairs from the end of the fragment that is 1753bp in size and 9 base pairs from the end of the fragment that is 2656bp in size. Due to the inability to check for proper removal of those ends, the digestion was assumed to have worked and then cleaned using a MinElute Reaction Cleanup kit from Qiagen. The cleaned and digested fragments were then ligated over a weekend, using ligase from Invitrogen Corporation. The result was the appearance of a faint band about 4300bp in size, which is the correct band size for the expected ligated product, which can be seen in figure 4. However, it appears most of the PCR fragments remained unligated, and each separate fragment could easily be seen on the gel at its respective size. In

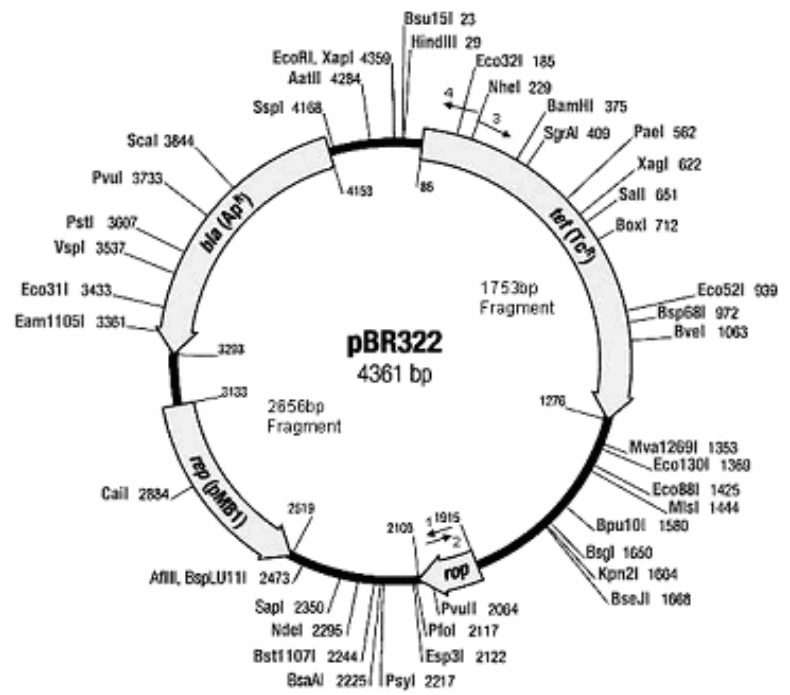

FIG. 2b The restriction map of the pBR322 plasmid. The location and direction of each of the primers (1, 2, 3, and 4) are shown on the map using an arrow. Therefore, primers 1 and 2 (the first set of primers) anneal to the *rop* gene and primers 3 and 4 (the second set of primers) anneal in the area of the *Nhe*I restriction site.

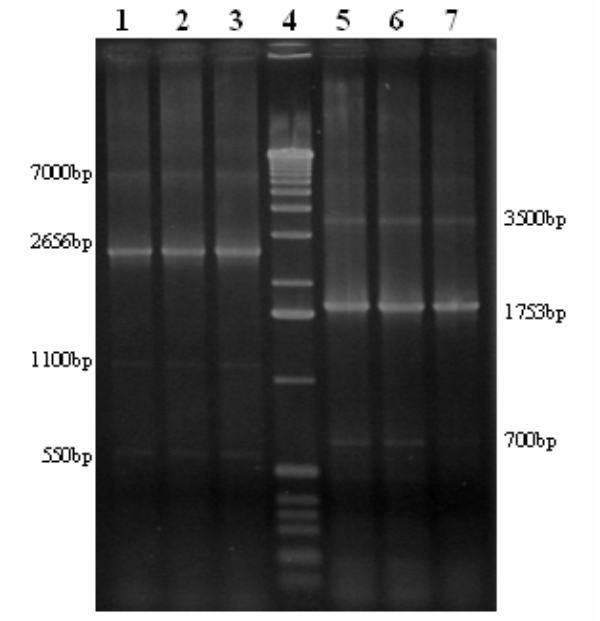

FIG. 3 Photograph of a 1.5% agarose gel loaded with the resulting PCR products. The PCR was carried out using all four primers. Lanes 1-3 are identical and show the fragments (2656bp in size) produced when primers 1 and 4 are used together and lanes 5-6 are identical and show the fragments (1753bp in size) produced when primers 2 and 3 are used together in a reaction. Lane 4 is the 1Kb DNA ladder.

addition, a large, smeared band could also be seen at a size that is greater than any of the bands in the 1Kb DNA ladder. Fortunately, since only 1/5 of the ligation reaction was run on the gel, there was a high possibility that there was enough ligated product present in the entire ligation reaction to be transformed into *E. coli* DH5α cells.

Transformations. The entire ligation reaction was separated into several different sets and then transformed into *E. coli* DH5α cells via electroporation and plated onto LB agar with 50μg/mL ampicillin. When DH5α cells electroporated with 5μL of DNA were plated, there was a large amount of growth. As the amount of DNA used in the electroporation decreased, the number of viable colonies found on the LB agar plates decreased in a similar manner. From the different plates, 36 colonies were chosen and grown in LB broth with both 50μg/mL ampicillin and 12μg/mL tetracycline. Twelve cultures survived the addition of tetracycline to the media and plasmid from each culture was isolated using the Gibco miniprep system.

Restriction Digest to check for mutations in *rop*. The primers were designed to include a unique enzyme restriction site, which is used to confirm the success of the addition of stop mutations within the *rop* gene. The assumption is that if the primer is incorporated into the plasmid and is able to generate a restriction site, then

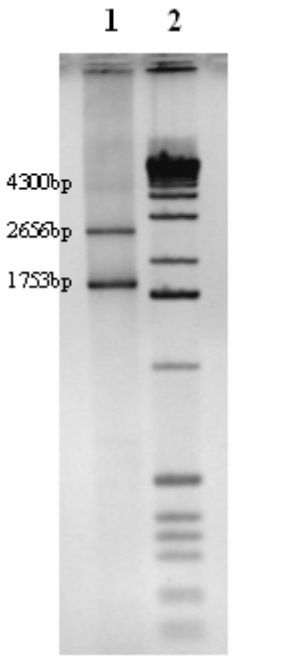

FIG. 4 Photograph of a 1.5% agarose gel loaded with the resulting ligation products in lane 1. Lane 2 is the 1Kb DNA ladder. The two most visible bands (1753bp and 2656bp) in lane 1 are the two fragments produced from the PCR reaction. The faint band at 4300bp is the potential ligated plasmid and the larger, smeared band above, is of an unknown product.

the desired stop mutations should also be generated (4). Using the plasmids isolated from the twelve cultures, *Kpn*I digests were performed. However, because the resulting bands appear to be the size of circular, supercoiled DNA and not cut, linear DNA, there did not appear to be a *Kpn*I site in any of the twelve plasmids. The digest was repeated multiple times using different digestion times, but no *Kpn*I sites were found in any of the twelve plasmids.

DISCUSSION

To obtain a plasmid with a mutagenic *rop* gene, PCR site-directed mutagenesis was attempted. Initially, with the use of two overlapping, complementary primers, the entire pBR322 plasmid was to be replicated, with the incorporation of the mutations and restriction sites into the *rop* gene using specifically designed primers. However, no PCR product was ever seen, even after multiple cycles of PCR. There are many possible reasons that could explain why the plasmid was never replicated. One primary reason may be the number of cycles used in the PCR reaction. Since the reaction was repeated multiple times with different variables, the number of cycles ranged from 16 to 40 cycles. This increased number of cycles may have in fact been the inhibiting factor in the reaction. The Stratagene QuikChange™ Site-Directed Mutagenesis Kit suggests using only 12-18 cycles (5) and members of UBC's Microbiology and Immunology Department suggest using only a few cycles such as 2-3 (W. Ramey, personal communication). The reason for this is that the polymerase and the circular plasmid DNA have no mechanism to dissociate each replicated DNA strand from the template. Therefore, the replication might not proceed in proportion to the number of cycles because of the entangled DNA, which may be one reason why no visible product is seen on the agarose gel. Another potential problem could be that the product is entangled. When the product is run on an agarose gel, no bands are visible and a transformation step would not occur. Therefore, if the agarose gel step is by-passed and the PCR product is transformed right away, since the product is not likely to be visible as a band, the entangled product would be transformed and eventually untangled into separate DNA strands, via intracellular mechanisms. Another possible solution may be to use a higher concentration of templates and to use fewer cycles, in order to allow a smaller clump of entangled DNA to be transformed and eventually untangled in the cell.

A second reason why this initial PCR reaction may not have worked may be that the primers were unable to anneal to the template since they were also complementary to each other, thus resulting in the chance of them annealing to themselves. If the primers could not anneal to the template then they would be unable to be elongated to produce a PCR product. However, the Stratagene QuikChange™ Site-Directed Mutagenesis process is still able to use analogus, opposing primers and that process seems to work. That process does not have a PCR portion; instead, the PCR products are transformed right away. If the primers did not anneal to each other, a third reason may be that the circular template was too supercoiled, so that as soon as the temperature decreased after the initial denaturation and uncoiling of the double-stranded DNA, the DNA re-annealed and coiled again. By re-annealing so quickly, there may not have been enough time provided for the primers to anneal to the template and keep the DNA denatured.

Although the initial attempts to replicate the entire pBR322 plasmid in one PCR reaction was shelved due to an inability to produce a product, a different method, which included producing two PCR fragments, was attempted. A second set of primers was designed so that they were about 1686bp away from the initial set of primers. Using a linearized plasmid, two primers, one from each set were used in each reaction, thereby producing two fragments. The resulting PCR products were designed so that the ends of each fragment overlapped the second fragment, and thus requiring a digestion step to produce sticky ends for ligation purposes. Therefore, there was no effective method to

ensure that the projected mutations had actually been incorporated into each fragment of the plasmid. Also, because so few bases were being removed from the ends of each fragment, there was no way of ensuring that the digestion had taken place. However, because the subsequent ligation step was so difficult, there was a large probability that the digestion may not have occurred at all or perhaps only a partial digestion occurred. When the ligation finally appeared to have worked, there was only a faint band, but because only a very small amount of DNA is necessary for a transformation, the ligation's product was transformed into competent
E. coli DH5α.

The twelve resulting cultures that were chosen had resistance to both ampicillin and tetracycline, which indicated that the ligation had been successful because each of the two fragments contained only one of the resistance genes to either ampicillin or tetracycline. Therefore, in order for the plasmid to be resistant to both antibiotics, the plasmid must contain both fragments. Finally, in order for the resistant genes to have been expressed, the plasmid must have been circularized, thus ligated together.
Each of the twelve cultures was then digested with *Kpn*I in order to check for the presence of the mutations in the plasmid. However, because the plasmids remained circularized, even after a *Kpn*I digestion, the mutagenesis was determined to not have been successful. In the digestion, controls had also been run so that it would be clear what size a supercoiled, circularized plasmid would be and what size a linearized plasmid would be on the specific gel used. There are several reasons why the primers and the expected mutations may not have been incorporated into the plasmid. One reason may be that the resulting PCR products were never digested with *Dpn*I to remove all parental template pBR322 plasmids. Therefore, there is a possibility that the plasmids transformed into the *E. coli* DH5α cells were actually just template DNA and not the separate PCR fragments ligated together. However, another real possibility could be that some PCR fragments did ligate, but the quantity was low so the 36 colonies that were picked did not correspond to the colonies with a ligated plasmid. Therefore, if more colonies were chosen, maybe one of the hundreds chosen would actually contain a colony with ligated DNA. The poor showing of ligated products is an indication of the extremely low efficiency rate of the ligation.

A second reason may be the presence of an unknown mechanism in the cells or on the pBR322 plasmid that selectively prevents this sort of mutagenesis from occurring. Therefore, the cell or plasmid may have some sort of mechanism that allows the plasmid to revert back to its original sequence. This is a possible reason because Matt Waldbrook's experiment (6) also had the same problem. When he sequenced the plasmid that should have been mutagenized, the plasmid did not appear to have had mutagenesis occur at all. Therefore, something else in the cell or on the plasmid may be happening that is preventing the mutagenesis in *rop* from occurring.

Technical challenges with the PCR reaction initially halted the progression of this experiment. However, with the design of a second set of primers, the PCR was successful in producing two fragments that needed to be ligated together in order to obtain a newly replicated pBR322 plasmid with mutations within its *rop* gene. Unexpectedly, the ligation step proved to be difficult, as the ligation rate of the two fragments into a circular plasmid was extremely inefficient. Therefore, the synthesis of a mutated pBR322 plasmid via site-directed mutagenesis was unsuccessful during the time allocated for this experiment and leaves many future experiments available for future researchers.

FUTURE EXPERIMENTS

There are several experiments possible that could address many of the questions and problems brought up in this study. Since only 36 colonies were picked and tested for the presence of ampicillin and tetracycline resistance, as well as a *Kpn*I site, more colonies could be chosen and tested for the presence of the necessary resistant components, as well as the unique restriction site.

Additionally, because there appears to have been a problem with the parental template strand population being in too high a quantity compared to the newly replicated DNA population, the PCR reaction using all four primers could be repeated with the addition of a digestion with *Dpn*I in order to digest and remove the methylated template DNA left in the PCR reaction. Following the digestion, the same procedure can then be followed, which would then include *Nhe*I and *Kpn*I digestions and finally ligation and transformation steps.

However, if there is still an issue with the ability to ligate the two PCR fragments together, the simplest solution would to be to use an alternative method that would bypass all the digestion and ligation steps. This method would be to attempt to use the initial site-directed mutagenesis method in this study. This one-step PCR method does not require the extra steps, because the entire pBR322 plasmid is replicated with the mutations already incorporated. As partially discussed in the previous discussion section, the initial PCR reaction could be repeated using fewer cycles. Rather than run a PCR reaction with 16-40 cycles, 2-3 cycles could instead be used in the reaction. Because only a few cycles are run, there is no point in checking for the success of the experiment; rather the finished

product of the PCR reaction should directly be digested with *Dpn*I to remove all remnants of the parental template DNA and then directly transformed into the *E. coli* DH5α cells. The cells can then be grown on either ampicillin or tetracycline plates in order to force the selection and growth of transformed cells. These cells will later be grown on media containing both ampicillin and tetracycline and then the plasmids within the cells should be isolated via minipreps. Once isolated, the plasmids could be checked for the presence of the unique *Kpn*I site using a digestion with *Kpn*I and *Nhe*I as a control. The presence of any cut, linear DNA in the resulting gel indicates a successful mutagenesis of the *rop* gene in pBR322 and should be followed with a co-transformation experiment with the wild-type pUC19 plasmid. However, sequencing should still be done in order to confirm the presence of the mutations and to ensure that extraneous mutations did not also arise during the PCR reaction.

Finally, if all other experiments fail to answer any questions, especially the question of *rop*'s role in pBR322, then new primers could be designed. Suitable new primers might increase the annealing rate of the primers to the template and allow better PCR reactions.

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