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Creation of a *Rop*⁻ Plasmid Using Site-Directed Mutagenesis

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pBR322 is a pColE1-derived plasmid that is widely used in molecular biology. Of particular interest is the observation that when pBR322 is co-transformed with pUC19, pBR322 is excluded from the cell. While many factors may contribute to this, it is the authors' opinion that the pBR322-encoded *Rop* protein is a major factor in this exclusion. *Rop* is a small protein that stabilizes the interaction between RNA I and RNA II, which in turns represses replication of pBR322. This study attempted to create a pBR322(Δ*Rop*) by inserting ochre codons into the *rop* reading frame, thus creating a non-functional *rop*, which would, theoretically, result in a higher copy number of pBR322. After the mutagenic PCR, ligation and transformation into DH5α cells, two clones appeared to possess the mutated plasmid, indicating that the mutagenic *rop* had correctly ligated into pBR322. Unfortunately, sequencing showed the two mutated clones had identical sequence to the wild-type *rop*, showing that the mutagenesis was unsuccessful.

Plasmid pBR322 is a 4361 bp plasmid that has been extensively used by molecular biologists as a cloning vector. It is derived from the plasmid pColE1, as are many other common plasmids. pColE1 and related plasmids all use a similar strategy for controlling replication. The plasmid encodes RNA II, which binds near the o*ri* and undergoes RNase H cleavage to yield a 3'-hydroxy at the replication start point. RNA I is an anti-sense message to RNA II, and when the two bind it prevents RNA II from initiating replication (1).

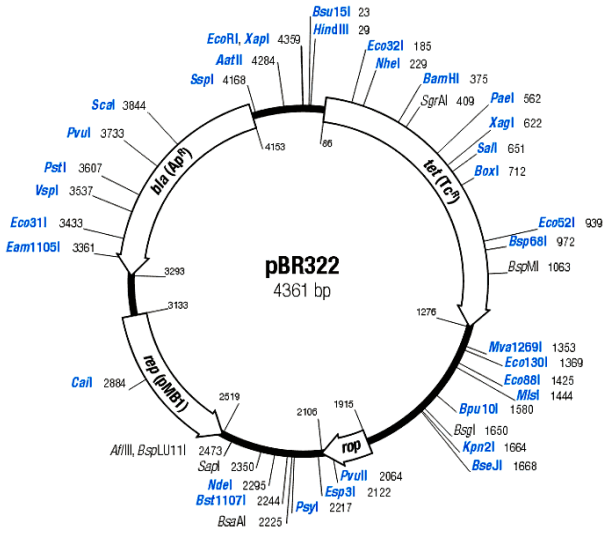

Figure 1. Map of pBR322

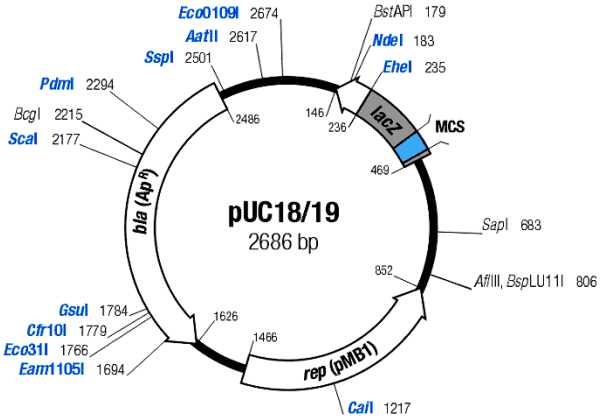

Figure 2. Map of pUC19

The small plasmid-encoded protein *rop* facilitates and stabilizes the interaction between RNA I and II. Thus, when there are many copy numbers of pBR322 present in the cell, there will be a correspondingly high level of *rop*, which in turn will further inhibit plasmid replication. With this simple mechanism, pBR322 is able to control plasmid replication when the copy number is high.

It has been observed that when pBR322 and another pColE1 derivative, pUC19, are transformed into the same cell, pBR322 is eventually eliminated, with approximately 90% of cells in the culture carrying only pUC19 (6). There are many possible explanations for this phenomenon:

1) pBR322 is 4361 base pairs in size, compared to pUC19's 2686 bp. Therefore, pBR322 will take a slightly longer time to complete the replication cycle. If a cell population containing both plasmids is rapidly dividing, then this discrepancy will eventually lead to cells containing only pUC19.
2) pUC19, as mentioned earlier, is derived from pColE1, and thus uses the same mechanism for replication control. However, the plasmid lacks the *rop* gene (2), meaning that replication is less efficiently repressed, as the RNA I and RNA II complex will dissociate more rapidly.
3) The sequence encoding the RNA II transcript in pUC19 contains a single point mutation, compared to the RNA II sequence in pBR322 (G 2975 in pBR322 to A 1306 in pUC19) (2). This mutation was found to inhibit the RNA I/II interaction (3). Therefore pUC19 has another mechanism to avoid the inhibitory effect of RNA I.

The focus of this study was to examine if the *rop* protein is involved in this exclusion effect. We hypothesize that if *rop* from pBR322 was non-functional or deleted, this would increase the copy number, and thus would not be excluded from the cell. To create this *rop*$^-$ plasmid, we decided to use a mutagenic approach to insert two ochre codons early in the *rop* reading frame. The reason why mutagenesis was used is that past attempts at removing the *rop* gene using restriction digests proved unsuccessful (4). Two mutations were planned in the PCR primers, in the chance that one did not mutate.

The experimental strategy involved performing the mutagenic PCR on pBR322, and ligating the product back into pBR322. The PCR generated an approximately 1100 bp fragment, containing the unique Eag I restriction site. Therefore, by digesting pBR322 with Eag I and Pvu II, and the PCR product with Eag I, we could easily ligate the two together (Eag I is a sticky end cutter). Also, since the PCR fragment ends near the mutagenic residues, the segment of *rop* from the primer binding site to the Pvu II cut site was removed, thus increasing the chance of producing a defective *rop.*

(T) (T)
GTGACC**A**AACAGGAA**A**AAACCGCCCTTAACATGGCCCGCTTTATCAGAAGCCAGACATTAAC
GCTTCTGGAGAAACTCAACGAGCTGGACGCGGATGAACAGGCAGACATCTGTGAATCGCTTC
ACGACCACGCTGATGAGCTTTACCG**CAG/CTG**CCTCGCGCGTTTCGGTGATGACGGTGAAAAC
CTCTGA *Pvu II*

Figure 3. *Rop* sequence and intended mutagenic alteration. This figure shows the *rop* reading frame sequence, from 5' to 3'. The bracketed residues show the intended mutagenic alterations, both of which result in the ochre (UAA) stop codon. Also shown is the location of the Pvu II cut site, which generates a blunt end fragment.

MATERIALS AND METHODS

PCR Reactions. Primer #1: 5'-GGAATCTTGCACGCCCTCGCTCAAGCCTTC-3', Primer #2: 5'-CCGGGTTTA TTCGTGTTAGGTCACTGA-3'. Reaction Conditions: 1 unit *Pfx* Polymerase enzyme, 0.3 mM dNTPs, 1X Plati-num *Pfx* Amplification Buffer, 1 mM $MgSO_4$, 0.3 μM Primer Mix (each primer), 100 ng pBR322 (template DNA). The reaction was conducted as follows, using Bio-Rad's Gene-Cycler PCR machine (5): 1st cycle: 2 min @ 94 ^oC, 30 sec @ 55^oC, 1 min @ 68^oC; 2nd-30th cycles: 15 sec @ 94 ^oC, 30 sec @ 55^oC, 1 min @ 68^oC.

Restriction Digest Conditions. *Eag*I (NEB) Digests: 1X NEB 3 Buffer, 0.5-1.0 μg DNA, 5 units *Eag*I Enzyme. *Pvu*II (Invitrogen) Digests: 1X REACT 6 Buffer, 0.5-1.0 μg DNA, 5 units Enzyme.

Ligation Reactions. Five microlitres Purified PCR Product, 0.5 μl Purified Digested pBR322, 4 μl 5x Ligase Buffer, 1 μl ATP (10 mM), 0.25 μl T4 Ligase (1 unit), 9.25 μl H_2O.

Sequencing Reactions. Sequencing Primers: #1: 5'-GCGAGGCAGCTGCGGTAAAGCT-3'; #2: 5'-GACTGCTG CCTGCAAAACGTCTG-3'. Sequencing Reactions and Gels performed by The Nucleic Acid and Protein Facility of The University of British Columbia, using Applied Biosystems' Big Dye v3.1 technology.

Producing DH5α Competent Cells. *Escherichia coli* DH5α cells were grown overnight in 5 ml LB Broth. This was used to inoculate 250 ml of LB Broth. The culture was grown at 37^oC, 200 rpm, until the culture reached a turbidity of approximately 0.7 (measured at 600 nm). Then, the culture was spun down (4000 x g, 15 min at 4^oC) and resuspended in 250 ml of cold, sterile 10% glycerol. The spin was repeated, and resuspended in 50 ml cold 10% glycerol. Finally, the culture was spun again and resuspended in 1 ml cold 10% glycerol. The culture was aliquoted into cold microcentrifuge tubes, 40 μl each, and stored at -80^oC.

Electroporation, using Bio-Rad's Micropulser Electroporation Device. Forty microliters of cold competent cells was pipetted into a cold electroporation cuvette. The cuvette was then placed into the device, and the Eco 1 program was run. Then, 1 ml of 37^oC LB broth was added to the cuvette, and the solution transferred to a sterile 14 ml Falcon tube, and allowed to grow at 37^oC for 1 hour. After the incubation, the solution was plated onto LB Ampicillin plates

RESULTS AND DISCUSSION

Mutagenic PCR. After reviewing the DNA sequence for *rop*, the primers for the mutagenic reaction were designed (refer to methods and materials for sequences). The primers were designed so that the PCR product contained the unique *Eag*I cut site, to allow for easy ligation of the product back into pBR322. The PCR product should be approximately 1100 bps, and should contain the two mutagenic alterations described in the Introduction, and the unique *Eag*I site should be approximately 30 base pairs from the 5' end of the product. Two samples of pBR322 were run at the same time, with exactly the same conditions, in case one reaction did not work.

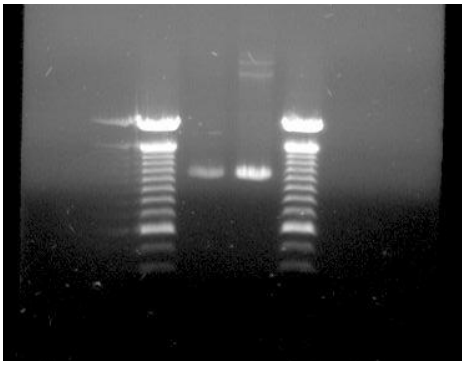

Figure 4. Gel Analysis of PCR Reaction. Five microlitres of the PCR Reaction was run on a 1% Agarose Gel. The DNA ladder is the 100 bp ladder (Invitrogen). The bright band is 600 bp. The approximate size of the PCR product is 1000 bp.

Even though the conditions were supposedly identical, the second reaction resulted in a larger quantity of DNA. This is probably due to a pipetting error that resulted in more template DNA being added to the mixture, as evidenced by the two larger bands in the lane. As the observed PCR product is approximately the same size as the expected product, we concluded that the mutagenic PCR was successful, and we would continue with the procedure.

Ligation and Transformation. DNA from the second PCR product was purified by gel extraction, in order to isolate the product from primer and template DNA. The remainder of the PCR reaction (approximately 40 μl) was run on a 1% gel, isolated, and purified using Qiagen's Gel Extraction kit.

Gel extraction resulted in a high loss of DNA, and the gel showed that very little of the PCR product remained after the process. Nevertheless, the product was pure, and there was no visible trace of contaminating template or primer DNA.

Next, pBR322 was prepared for ligation with the PCR product. A double digest of Eag I and Pvu II was performed overnight on 2 μg of plasmid DNA. The next day, the digest was run on a 1% agarose gel and the band at approximately 3.0 kbp was excised and purified (data not shown). Also, the PCR product was digested with Eag I; to allow more efficient ligation between the plasmid and the PCR product (the PCR digestion was not gel purified, as there was little DNA to begin with).

The digested product and plasmid were ligated overnight at approximately 14 ^{o}C. The following day 2 μl of the ligation mixture was electroporated into competent DH5α cells. The cells were initially plated onto LB-Ampicillin plates. We rationalized that if the PCR product ligated incorrectly into the plasmid, this would disrupt the Tet^{R} gene. We can therefore use Tetracycline as a screening agent to determine if cells possess the properly inserted PCR product.

After the overnight incubation, the LB-Amp plates showed 10 colonies. These in turn were patched onto a LB Amp + Tet plate. After incubation, only two colonies showed Tetracycline resistance. Since our ligation reaction was half-blunt end and half-sticky end, we suspected that the ligation would be less efficient than a two sticky end ligation. Therefore, two colonies showing Tetracycline resistance out of ten was thought to be in accordance to the probability of the correct ligation.

Sequencing. The two clones isolated from the Tetracycline plate (named clone 1 and clone 6) were sequenced by The Nucleic Acid and Protein Facility (UBC). The sequencing primers were designed to place the *rop* sequence approximately halfway through the sequence, which would allow for maximum resolution between nucleotides.

The sequences were then taken and aligned with the wild type *rop* sequence obtained from NCBI (http://www.ncbi.nlm.nih.gov/). We intended to sequence the pBR322 plasmid initially used in the experiment, and

use that sequence as our wild-type data. However, no signal was detected during the process, and we had insufficient time to repeat the sequencing. Alignment determination used the DIALIGN2 program, available from the Pasteur Institutes web site (http://www.pasteur.fr/English.html).

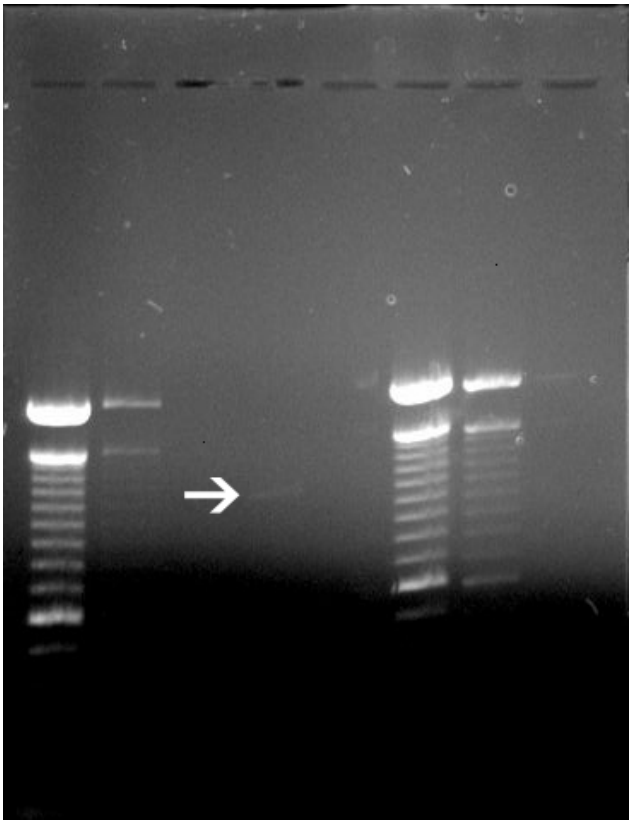

Figure 5. Analysis of Gel Purified PCR Product. The gel extract (5 μl) was run on a 1% agarose gel. The ladder is a 100 bp ladder (duplicate ladders in adjacent lanes are due to spillover).

WT gctcatCAGC GTGgtCGTGA AGCgattCAC AGATGTCTGC CTGTTCATCC
Clone 1, Primer 1 ------CAGC GTGn-CGTGAAGCctn-CAC NGATGTCTGC CTGTTCATCC
WT GCGTCCAGCT CGTTGAGTTT CTCCAGAAGC GTTAATGTCT GGCTTCTGAT
Clone 1, Primer 1 GCGTCCAGCT CGTTGAGTTT CTCCAGAAGC GTTAATGTCT GGCTTCTGAT
WT AAAGCGGGCC ATGTTAAGGG CGGTTTT**T**TTC CTGTT**T**GGTC AC--------
Clone 1, Primer 1 AAAGCGGGCC ATGTTAAGGG CGGTTTT**T**TTC CTGTT**T**GGTC ACtgatgcct

WT ---------- ---------- GTGACC**A**AAC AGGAA**A**AAAC CGCCCTTAAC
Clone 1, Primer 2 ccccttacac ggaggcatca GTGACC**A**AAC AGGAA**A**AAAC CGCCCTTAAC
WT ATGGCCCGCT TTATCAGAAG CCAGACATTA ACGCTTCTGG AGAAACTCAA
Clone 1, Primer 2 ATGGCCCGCT TTATCAGAAG CCAGACATTA ACGCTTCTGG AGAAACTCAA
WT CGAGCTGGAC GCGGATGAAC AGGCAGACAT CTGTGAATCG CTTCACGACC
Clone 1, Primer 2 CGAGCTGGAC GCGGATGAAC AGGCAGACAT CTGTGAATCG CTTCACGACC
WT ACGCTGATGA GCTTTACCGC AGCTGCCTCG CGCGTTTCGG TGATGACGGT
Clone 1, Primer 2 ACGCTGATGA GCTTTACCGC AGCTGCCTCG CGCGTTTCGG TGATGACGGT
WT GAAAACCTCT GA-------- ---------- ---------- ---------- ----------
Clone 1, Primer 2 GAAAACCTCT GAcacatgca gctcccggag acggtcacag cttgtctgta

Figure 6A. Sequencing Results from Clone #1

WT	gctcatCAGC GTGG tcgtga agcgattCAC AGATGTCTGC CTGTTCATCC
Clone 6, Primer 1	------CAGC GTGGcgtgaa cctn----CAC AGATGTCTGC CTGTTCATCC
WT	GCGTCCAGCT CGTTGAGTTT CTCCAGAAGC GTTAATGTCT GGCTTCTGAT
Clone 6, Primer 1	GCGTCCAGCT CGTTGAGTTT CTCCAGAAGC GTTAATGTCT GGCTTCTGAT
WT	AAAGCGGGCC ATGTTAAGGG CGGTTT**T**TTC CTGTT**T**GGTC AC--------
Clone 6, Primer 1	AAAGCGGGCC ATGTTAAGGG CGGTTT**T**TTC CTGTT**T**GGTC ACtgatgcct

WT	---------- ---------- ---------- ---- GTGACC **A**AACAGGAA**A**
Clone 6, Primer 2	catgaacaga aatccccctt acacggaggc atcaGTGACC **A**AACAGGAA**A**
WT	AAACCGCCCT TAACATGGCC CGCTTTATCA GAAGCCAGAC ATTAACGCTT
Clone 6, Primer 2	AAACCGCCCT TAACATGGCC CGCTTTATCA GAAGCCAGAC ATTAACGCTT
WT	CTGGAGAAAC TCAACGAGCT GGACGCGGAT GAACAGGCAG ACATCTGTGA
Clone 6, Primer 2	CTGGAGAAAC TCAACGAGCT GGACGCGGAT GAACAGGCAG ACATCTGTGA
WT	ATCGCTTCAC GACCACGCTG ATGAGCTTTA CCGCAGCTGC CTCGCGCGTT
Clone 6, Primer 2	ATCGCTTCAC GACCACGCTG ATGAGCTTTA CCGCAGCTGC CTCGCGCGTT
WT	TCGGTGATGA CGGTGAAAAC CTCTGA---- ---------- ----------
Clone 6, Primer 2	TCGGTGATGA CGGTGAAAAC CTCTGAcaca tgcagctccc ggagacggtc

Figure 6B. Sequencing Results from Clone #6

In each of the sequences, the bold nucleotides indicate the intended mutagenic changes. As the sequences show, there are no changes between the clones and the wild type sequence. The creation of a *rop*$^-$ plasmid was unsuccessful. Most likely this resulted during the ligation of the PCR product into the cut pBR322. It is possible that uncut pBR322 survived the digestion and was purified along with the cut pBR322. Thus, when the cells were transformed with the ligation mixture, it is possible that the cells acquired normal, unaltered pBR322. These cells would show Ampicillin and Tetracycline resistance, and would probably be a small proportion of plasmids in the ligation reaction. Therefore, a small proportion of the total transformants would carry the unaltered pBR322, and just by chance happened to be the colonies selected for sequencing.

Another possibility is that the resulting plasmid was deficient in some way that prevented proper replication of the plasmid, thus selecting for the wild type plasmids. Since we know that RNA I and RNA II are directly responsible for plasmid replication, we thought that perhaps the missing segment of *rop* contained a promoter or a part of the RNA I or RNA II gene. However, analysis of the pBR322 sequence showed that the origin and coding region for these molecules lays approximately 1 kbp's upstream of the *rop* gene. Therefore, it is unlikely that interfering with the expression of these molecules are responsible for the observed results.

The last likely explanation may be because there will be a small number of plasmid molecules that are unaltered during the PCR reaction; the plasmid template molecules. Again, it is possible that these molecules were purified with the PCR product, and transformed into the cells, giving the wild-type sequence results. While

CONCLUSIONS

The attempt to create a *rop*$^-$ plasmid using mutagenesis did not result in a plasmid with a defective *rop*. The two clones isolated from the procedure had identical sequences to the wild type *rop*. Future work on this matter will examine the colonies that show Ampicillin resistance but not Tetracycline resistance. This will show if those colonies bear the mutagenized *rop*, but the PCR product ligated incorrectly into pBR322, thus disrupting the *tet*^{R} gene. We will then know if the mutagenic PCR was successful but the following ligation is an improbable event. This analysis should be possible with careful restriction analysis, as the restriction sites will be different between the wild type and ligated plasmids. If not, sequencing would be the only other option.

ACKNOWLEDGMENTS

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