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Construction of pCAWK, a Novel pBR322-derived Plasmid with Insertional Inactivation of the *rop* Gene

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The ColE1-type plasmid pBR322 is commonly used as vector tool in molecular biology. It has been observed that when *Escherichia coli* cells are co-transformed with pBR322 and pUC19 (another ColE1-type plasmid) pUC19 is retained and pBR322 is excluded. Several mechanisms have been proposed to explain this phenomenon, including the larger size of pBR322, absence of the *rop* gene in pUC19, and an existing point mutation in RNA II of pUC19 absent in pBR322. To examine the role of the repressor of primer (Rop) protein on the exclusion effect, previous studies have attempted to create a non-functional Rop protein by PCR-site directed mutagenesis but have been unsuccessful. The objective of this study was to create a Rop mutant by insertional inactivation of the *rop* gene. A 34-bp DNA insert was designed with (1) PvuII restriction digest sites flanking the ends; (2) one stop codon in each of six possible reading frames; and (3) a central EagI restriction site. This DNA was ligated into the *rop* gene of pBR322 so that Rop protein translation is terminated prematurely after the 50th amino acid, arginine. This disruption will result in a non-functional protein as it has been demonstrated that residues Phe-56 and Gly-57 are essential for Rop functionality. Restriction digests confirmed successful insertion of the DNA insert into the *rop* gene to create mutant pBR322. This study was the first to be successful in creating a mutant pBR322 plasmid with a disrupted *rop* gene, named pCAWK. This novel plasmid can be used to further explore the role of the Rop protein on pBR322 exclusion.

Plasmid pBR322 is a widely used multipurpose cloning vector in molecular biology, and a large number of pBR322 derivatives have been created for specific applications and research purposes (1). The vector carries the genes for tetracycline and ampicillin resistance, repression of primer (Rop), and the origin of replication. It is derived from the natural isolate pMB1 and its origin of replication shows a strong homology to several other origins found in natural isolates (1). Collectively, these origins are called ColE1-type origins (1).

The replication process of pBR322 is complex and the mechanism of regulation involves several different components. Replication is initiated when primosome and replisome structures assemble at the specific assembly sites, downstream of the replication origin (1). Following the assembly, a 550 nucleotide-long, single-stranded RNA called RNA II is transcribed from a region known as promoter 2 (1) and later cleaved by RNase H, allowing it to serve as a primer for DNA polymerase I for the synthesis of the leading strand (1). This regulation of replication process is countered by a 108 nucleotide antisense RNA molecule called RNA I (1). RNA I is transcribed from a region known as promoter 1 and it is expressed in 100-fold excess relative to RNA II molecule (1).The hybridization of RNA I to RNA II prevents RNA II from acting as a primer and thus replication is disrupted. It is the Rop protein that facilitates this RNA I-RNA II interaction, thus limiting copy number (1).

It has been observed that when pBR322 is co-transformed with pUC19 into *Escherichia coli* cells, only pUC19 is retained and pBR322 is excluded (6). Several possible explanations for this phenomenon have been discussed (16). First of all, pBR322 is a larger plasmid compared to pUC19, 4361 bp and 2686 bp, respectively, which presents as a disadvantage for pBR322 since it takes a slightly longer time to finish one replication cycle, and over time, it will be present in a lower copy number relative to pUC19 (16). In addition, pBR322 contains the *rop* gene, which is absent in pUC19 (3). Therefore, pUC19 exhibits a weaker RNA I-RNA II interaction, resulting in a higher copy number (1). Furthermore, pUC19 contains a G→A point mutation in RNA II, which may prevent efficient binding of RNA I (7) and again, result in a higher copy number (9).

These observations have led to the hypothesis that

with the creation of a *rop* gene mutant, pBR322 copy number would increase, which will consequently increase the probability of its retainment in *E. coli* cells during co-transformation with pUC19. Many unsuccessful attempts have been made to create a mutation in the *rop* gene by PCR site-directed mutagenesis to investigate the role of Rop protein in the exclusion effect of pBR322 upon co-transformation with pUC19 (3, 7, 8, 16). Since this was ineffective in creating a mutation, this study attempted a different approach.

The focus of this study was to disrupt the *rop* gene by insertional inactivation. The Rop protein is an acidic, 63-residue-long protein that functions as a dimer (1) and is coded by the 191 bp *rop* gene (1915 to 2106) in pBR322. Within this gene, there are two restriction digest recognition sites: PvuII and XmnI. PvuII is a unique restriction digestion site at 2064, whereas there are two XmnI restriction digest sites in pBR322. Therefore, we chose to use the PvuII restriction endonuclease to cut within the *rop* gene (Fig. 1). We designed double-stranded DNA that contains: 1) PvuII restriction sites that flank the ends; 2) one stop codon in each of the 6 possible reading frames; and 3) a central EagI restriction site. PvuII-digested pBR322 was ligated with the insert DNA and used to transform *E. coli* cells via electroporation. Following selection for transformed cells with ampicillin, four colonies at random were selected and their plasmid DNA was extracted. Restriction digestion of isolated plasmids with EagI revealed that the insert DNA had ligated successfully with pBR322. The modified plasmid was named pCAWK and can be used to further explore the role of the Rop protein on pBR322 exclusion.

MATERIALS AND METHODS

Restriction digest of pBR322 DNA. A restriction digest of pBR322 (Fermentas, #SD0041) with PvuII, recognition site 5'-CAG/CTG- 3' (Fermentas, #ER0631) was performed according to the manufacturer's protocol. The reaction mixture was incubated for 16 hours at 37°C and was stopped with the immediate purification of digested pBR322.

Purification of PvuII digested pBR322. The GeneJet PCR Purification Kit (Fermentas, #K0701) was used to purify PvuII digested pBR322 from the rest of the restriction digest reaction mixture, according to the manufacturer's protocol. All centrifugation steps were performed at 12,000 x g for 1 minute on a tabletop microcentrifuge (Eppendorf AG, Centrifuge 5415 D). The purified DNA concentration was measured by UV spectroscopy (NanoDrop 2000c Spectrophotometer, Thermo Scientific) and stored at -20°C.

Gel electrophoresis visualization of linearized pBR322. The PvuII digested pBR322 plasmid was visualized on a 0.8% (w/v) agarose gel (BIO-RAD, 161-3101) with 1X Tris-acetate-EDTA (TAE) Buffer (400 mM Tris-base, 1% v/v glacial acetic acid, 10 mM EDTA, pH 8.0, in 1 L of distilled water, pH 8.0). To each DNA sample, 6X DNA loading dye (Fermentas, #R0611) was added. One µg of the GeneRuler 1 kb DNA Ladder (Fermentas, #SM0311) was used. The gel was run for 45 minutes at 120 V and post-stained in an ethidium bromide bath (0.5 µg/ml) for 20 minutes. The sizes of PvuII digested and supercoiled pBR322 were compared on the gel to assess whether the restriction digest reaction was successful.

Dephosphorylation reaction. PvuII digested pBR322 was dephosphorylated with Calf Intestinal Alkaline Phosphatase (New England Biolabs, #M0290S) according to the manufacturer's protocol, in order to reduce the possibility of plasmid religation. The reaction mixture was incubated for 60 minutes at 37°C and stopped with the immediate purification of pBR322.

Purification of PvuII digested and dephosphorylated pBR322. The GeneJet PCR Purification Kit (Fermentas, #K0701) was used to isolate PvuII digested and dephosphorylated pBR322 from the rest of the dephosphorylation reaction mixture, according to the manufacturer's protocol. The purified DNA concentration was measured was measured by UV spectroscopy (NanoDrop 2000c Spectrophotometer, Thermo Scientific) and stored at -20°C.

DNA insert. Double-stranded insert DNA (Fig. 2) synthesized by Integrated DNA Technologies (IDT), was designed to be compatible on both the 5' and 3' ends with PvuII digest sites on pBR322, phosphorylated on the 5' end (to allow for ligation with dephosphorylated pBR322) and to contain 6 stop codons (1 in each of the 6 possible open reading frames), in addition to a central Eco52I/EagI recognition site (5'-/5Phos/CTGGATCGATCGATCGGCCGTAGCTAGCTAGCAG-3').

Ligation reaction of pBR322 and insert DNA. One reaction was performed following a Fermentas protocol and one reaction was performed following an Invitrogen protocol. This set-up constituted a blunt-end ligation reaction, and therefore maximum ligation efficiency was important. For the Invitrogen protocol, reaction conditions were set-up according to the manufacturer's protocol supplied with T4 DNA Ligase (Invitrogen, #15224-025). For the Fermentas protocol, reaction conditions were set-up according to the manufacturer's protocol supplied with T4 DNA Ligase (Fermentas, #EL0011) except that PEG 4000 was excluded.

Purification of plasmid from the ligated reaction of pBR322 and insert DNA. The GeneJet PCR Purification Kit (Fermentas, #K0701) was used to isolate plasmid DNA from the rest of the ligation reaction mixture, according to the manufacturer's protocol. The purified DNA concentration was measured by UV spectroscopy (NanoDrop 2000c Spectrophotometer, Thermo Scientific) and stored at -20°C.

Preparation of cells for transformation. A protocol from "Molecular Cloning - A Laboratory Manual" (13) was followed to prepare *E. coli* DH5α cells for transformation. This strain was obtained from Dr. Ramey from the Microbiology and Immunology Department at the University of British Columbia. All centrifugation steps required were performed with the IEC Centra MP4R Benchtop Centrifuge Refrigerated High Speed, at 1000 x g and 4°C for 20 minutes. Cells were grown in Luria Broth at 37°C on a rotary shaker (250 rpm) until an OD_{600} of 0.4 was reached. Immediately, bacteria were placed on ice for 30 minutes. Four centrifugation steps were performed, each time resuspending the cell pellets, first with 500 ml of distilled water, second with 250 ml of 10% glycerol (BDH Molecular Biology Reagents, B28454-76), third with 10 ml of 10% glycerol and finally with 1 ml of ice-cold GYT medium (10% v/v glycerol, 0.125 % w/v yeast extract and 0.25 % w/v tryptone). Next, the cell suspension was diluted to 3 x 10^{10} cells/ml (assumption of 1 OD_{600}= 2.5 x 10^8 cells) with ice-cold GYT medium and used immediately for electroporation.

Electroporation. A protocol from "Molecular Cloning - A Laboratory Manual" (13) was followed to transform the electrocompetent *E. coli* DH5α cells with the pBR322 DNA insert ligation reaction mixture. To 40 µl of cell suspension, varying amounts of plasmid DNA (9 ng, 94 ng and 179 ng) were added, 3 of

which were from the Fermentas protocol ligation reaction and 3 of which were from the Invitrogen ligation reaction. In 0.2 cm cuvettes, a pulse of electricity was delivered to the cells with the Micropulser Electroporation Apparatus (BIO-RAD, #165-2100) with program EC2 for *E. coli* (4-5 milliseconds, 2.5 kV, 200 ohm resistance) and incubated into 1 ml of SOC medium (20 g tryptone, 5 g yeast extract, 0.5 g of NaCl, 10 ml of 250 mM KCl, pH 7.0 in 1 L of nuclease free water and 20 mM of glucose) at 37°C on a 250 rpm rotary shaker for 1 hour. Next, 200 µl of the culture was plated onto SOB medium containing 100 µg/ml ampicillin sterilized with a 0.22 µm filter (Millipore, GSWP02500) and incubated at 37°C on a 250 rpm rotary shaker for 30 hours.

Enrichment of transformed *E. coli* DH5αcells. Four colonies at random were selected from 4 different SOB plates, 2 of which were transformed with plasmid DNA from the Fermentas ligation reaction protocol and 2 of which were transformed with plasmid DNA from the Invitrogen ligation reaction protocol. Colonies were each streaked onto SOB medium plates containing 100 µg/ml ampicillin and also inoculated into 10 ml of LB (10 g tryptone, 5 g yeast extract and 10 g NaCl in 1 liter of distilled water) containing 100 µg/ml ampicillin and incubated at 37°C on a 250 rpm on a rotary shaker for 30 hours.

Plasmid isolation from transformed *E. coli* DH5α cells. Plasmids from the transformed *E. coli* DH5α cells were isolated by the GeneJET Plasmid Miniprep Kit (Fermentas, #K0503) using the protocol provided by the manufacturer. All centrifuges were performed on a tabletop microcentrifuge (Eppendorf AG, Centrifuge 5415 D) at room temperature. The purified DNA concentration was measured by UV spectroscopy (NanoDrop 2000c Spectrophotometer, Thermo Scientific) and stored at -20°C.

Restriction digest of isolated plasmid DNA. Isolated plasmids derived from the 4 different transformed *E. coli* DH5α isolates were digested with Eco52I/EagI, recognition site 5' -C/GGCCG- 3' (Fermentas, #ER0331) according to the manufacturer's protocol. The reaction mixture was incubated for 16 hours at 37°C and the restriction endonuclease was then inactivated by incubating the reaction mixture at 65°C for 20 minutes.

Gel electrophoresis visualization of Eco52I/EagI digested plasmids. The Eco52I/EagI digested plasmids isolated from transformed colonies were visualized on a 0.8% (w/v) agarose gel with 1X TAE Buffer (pH 8.0). To each DNA sample, 6X DNA loading dye (Fermentas, #R0611) was added. One lane contained 1 µg of the GeneRuler 1 kb DNA Ladder. The gel was run for 45 minutes at 120 V and post-stained in an ethidium bromide bath (0.5 µg/ml) for 20 minutes. The sizes of Eco52I/EagI digested isolated plasmids, Eco52I/EagI digested pBR322 and supercoiled pBR322 were compared on the gel to identify the isolate containing the pBR322, which encompasses the DNA insert in the *rop* gene.

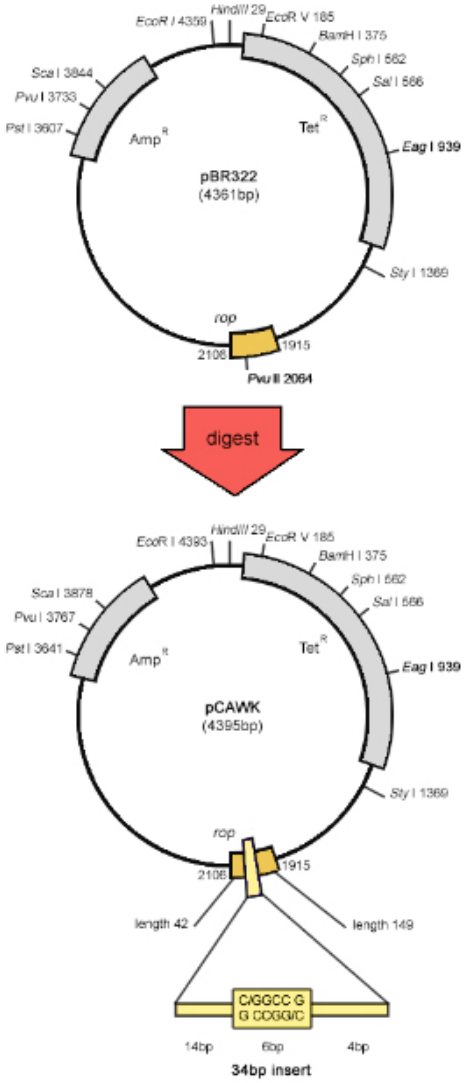

FIG. 1. pBR322 and novel plasmid pCAWK. A 34-bp insert containing a 6-bp central Eco52I/EagI site flanked by stop codons is ligated into the PvuII restriction site.

RESULTS

pBR322 digestion with PvuII. pBR322 plasmid obtained from Fermentas was digested with the restriction enzyme PvuII. PvuII recognizes position 2064 bp in pBR322 (Fig. 2). As predicted, digestion with this restriction endonuclease resulted in the appearance of a band at approximately 4361 bp on an agarose gel (Fig. 3). The linearization of the plasmid was confirmed by comparison to the size of the supercoiled undigested pBR322 control, which, as expected, migrated faster than the linearized form on the agarose gel (data not shown).

Ligation of insert DNA into *rop* gene and transformation. The premature stop codons present in the insert DNA should ensure termination of amino acid elongation after the 50th amino acid arginine is attached to the growing polypeptide, and as it has been demonstrated that Phe-56 and Gly-57 or both are essential for Rop functionality, this should create a non-functional Rop protein (2). The insert DNA was ligated into PvuII digested and calf-intestinal phosphatase dephosphorylated pBR322 using protocols from both Invitrogen and Fermentas. The Invitrogen protocol included PEG in the ligation reaction, as opposed to the

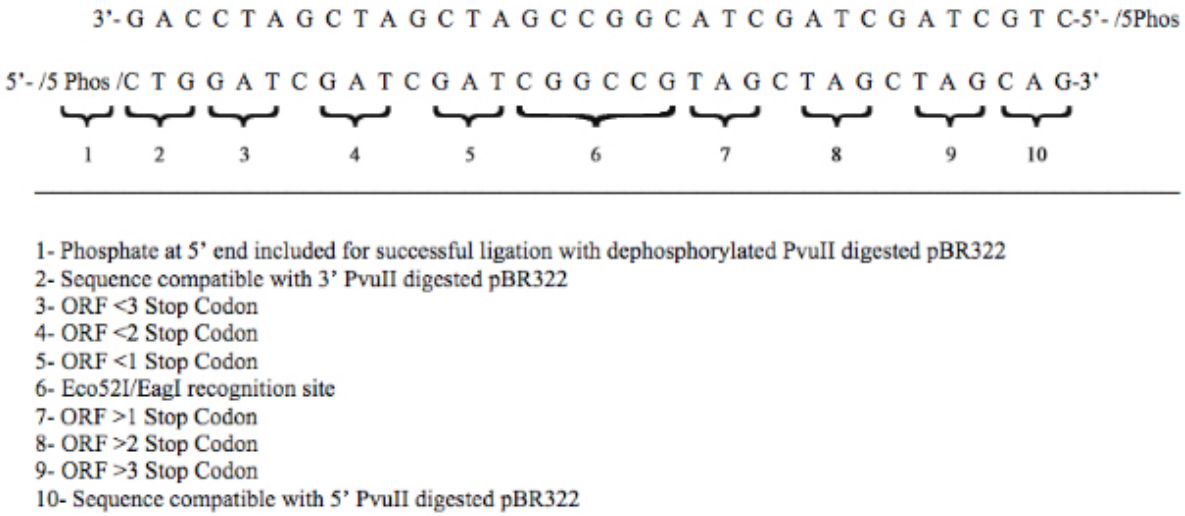

FIG. 2. Double-stranded oligonucleotide insert design for insertion into the *rop* gene of pBR322.

modified Fermentas ligation protocol. PvuII digestions leave blunt ends, which is inefficient in ligation. Since PEG has been previously shown to increase blunt-end ligation efficiency, but to reduce transformation efficiency (5), both protocols were utilized to compare the success of transformation with the plasmid of interest (pBR322 containing insert DNA). Different concentrations of plasmid DNA were used in electroporation to increase the probability of success with transformation (9 ng, 94 ng and 179 ng). No difference was observed in the number of transformants resulting from the Invitrogen and Fermentas protocols, as plasmids from both ligation protocols yielded more than 300 colony forming units per plate. However, there was relatively more transformants per plate when a higher concentration of plasmid DNA was used in electroporation for both protocols.

Restriction digest to confirm incorporation of DNA insert. *E. coli DH5α* cells transformed with mutant pBR322 were enriched using media containing ampicillin, to select for the transformants. Four colonies at random from 4 different plates were picked for further analyses to locate the transformants containing the insert DNA in pBR322. Plasmids were extracted using the GeneJet Plasmid purification kit and digested for 16 hours with Eco52I/EagI restriction endonuclease. Results from the visualization of the resulting bands on an agarose gel are shown in Fig. 3. As expected, the undigested pBR322 migrated faster than the digested pBR322 control (Fig. 3, lanes 2 and 3). As demonstrated in Fig. 2, pBR322 contains an Eco52I/EagI restriction site at 939 bp, and the insert DNA contains a second Eco52I/EagI restriction site at 2083 bp. Therefore, isolated plasmids from *E. coli DH5α* transformants containing the DNA insert were expected to yield two bands with the molecular sizes of 3251 and 1144 bp after digestion with this restriction endonuclease. Results from Fig. 3 (lanes 4 – 7) show two bands of expected sizes in each lane, confirming the integration of the DNA insert into pBR322 in those four isolated colonies. This is further validated with the digested pBR322 control (lane 3), demonstrating a lone band at 4361 bp resulting from the linearization of the plasmid at the only one Eco52I/EagI restriction site. It should be noted that the DNA insert was not designed to be palindromic and thus the orientation of the insert affects the expected size of the fragments resulting from Eco52I/EagI digestion by five nucleotides (Fig. 2). Therefore, the Eco52I/EagI recognition site within the DNA insert could be either 1144 or 1140 nucleotides away from the Eco52I/EagI recognition site of pBR322, resulting in bands of either 1140 and 3255 or 1144 and 3251. As visualization through an agarose gel does not allow for the differentiation of these two possibilities, this difference was not addressed in this paper.

It appears the 1144 fragments are approximately 2X less intense than the 3251 fragments of the Eco52I/EagI digested plasmid (Fig. 3, lanes 4-7). This is surprising as a 1:1 ratio of the fragments should be present, and therefore, equal intensities of the fragments were expected. However, it has previously been shown that one ethidium bromide molecule intercalates at every 4-5 nucleotides in DNA, and then a secondary binding process occurs, resulting in the saturation of all

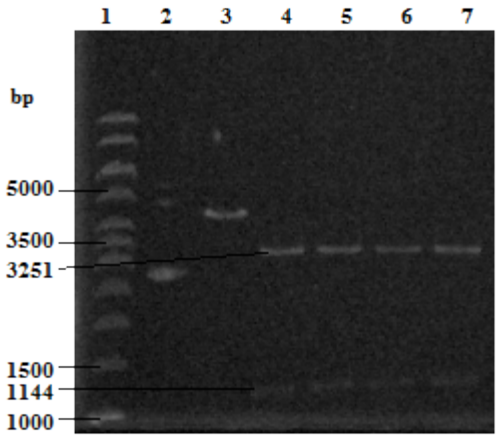

FIG. 3. Eco52I/EagI restriction enzyme digest analysis of selected colonies to verify insert. Lane 1: 1 kb DNA ladder, lane 2: undigested pBR322, lane 3: pBR322, lanes 4 and 6: Eco52I/EagI digested plasmids from transformed colonies using Fermentas protocol, lanes 5 and 7: Eco52I/EagI digested plasmids from transformed colonies using Invitrogen protocol.

nucleotide sites (17). Therefore, it is possible that the smaller fragment appears less intense because there would be 423-527 less ethidium bromide molecules intercalated between it than the larger fragment.

DISCUSSION

Previous experiments have attempted to disrupt the *rop* gene to study the role of the Rop protein in the exclusion effect observed between pUC19 and pBR322 upon co-transformation. Many of these experiments attempted to use site-directed mutagenesis to achieve this. This study aimed to use insertional inactivation by introducing a DNA insert into the *rop* gene of pBR322.

Plasmids were extracted from the transformed *E. coli* pBR322-insert DNA ligation reaction and digested with Eco52I, and bands of 3251 bp and 1144 bp were observed (Fig. 3), indicating successful ligation of our insert, which contains a second Eco52I site, into pBR322. The presence of the second Eco52I recognition site within the expected *rop* gene also indicates the presence of the premature stop codons at a location expected to disrupt the *rop* gene. The principle demonstrated by Fig. 3 is that insertional inactivation as described in this paper is a valid method for the disruption of gene sequences. This addresses the shortcomings of strategies employed by previous authors who were not successful in altering the *rop* gene through site-directed mutagenesis (3, 7, 16). Both attempts encountered difficulties related to ligation of PCR products containing mutated segments (3, 7, 16). Waldbrook's attempt to insert ochre codons by site-directed mutagenesis and ligation into pBR322 failed to produce plasmids containing the ochre codons (16). This was attributed to contaminating unmutated pBR322 or incorrect ligation. In Fang's strategy, failure to produce mutated pBR322 was attributed to the use completely overlapping primer pairs and formation of primer-dimers (3, 7).

It should be noted that the insert is not palindromic. While the design of the insert provides stop codons in each reading frame and both orientations (Fig. 2), the orientation in which the insert ligated may have an effect on the degree of disruption of *rop*. When introducing mutated sequences into a plasmid the direction of insertion is often important. Insertion in one orientation may knock out a gene with no polar effects, while the opposite orientation exerts polar effects (18). However, our insert was designed to have stop codons in all possible reading frames and orientations. Therefore, though different orientations may produce some difference in mitigating the exclusion effect, it is unlikely to produce a notable effect.

In order to preserve the size of the pBR322 plasmid, no reporter gene could be inserted with our construct. However, the high number of transformants observed (data not shown) provided sufficient colonies carrying the target novel plasmid (Fig. 3). In this experiment, 1.2 x 10^9 cells were transformed with 9 ng, 94 ng, or 179 ng of plasmid DNA from the ligation reaction following both the Fermentas and Invitrogen protocols. Relative transformation is expected to decrease around an optimal 1.6 x 10^8 cells per 1 ng plasmid DNA as the plasmid:cell ratio is changed, with efficiency more greatly reduced with a higher ratio than lower (11, 14). However, our results showed no measurable difference in transformation efficiency among the three various concentrations, with plasmid:cell ratios of 1.71 (9 ng), 17.87 (94 ng), and 34.0 (179 ng). These results follow observations made by Hanahan (4) where such ratios yielded similar numbers of transformed cells. No differences were observed in transformation efficiency between the Invitrogen and modified Fermentas protocols for ligation (data not shown). The hydrophobic PEG is often added to improve ligation by increasing the effective concentrations of DNA and T4 ligase. Ligase and PEG produces vector-and-insert oligomers (12), which are then cleaved by the restriction endonuclease to form monomers that recirculize (15). However, PEG can decrease transformation efficiency if heat inactivated (10). Therefore, any positive effect that PEG

had on ligation appears to have been balanced by any negative effects on transformation efficiency, therefore both methods yielded similar results. It is likely that both Invitrogen and Fermentas kits are sufficiently optimized such that an absence of PEG in the Fermentas method is negligible, and yield similar results.

This study aimed to use insertional inactivation by introducing a DNA insert into the *rop* gene of pBR322, which is expected to terminate translation due to the presence of stop codons. Results indicate that insertional inactivation was successful in disrupting the *rop* gene in pBR322. This mutant pBR322 plasmid , named pCAWK, will be useful for exploring the role of the Rop protein on pBR322 exclusion.

FUTURE DIRECTIONS

We have succeeded at creating a pBR322 derived plasmid by disruption of the *rop* gene. However, this plasmid has not been fully characterized yet as the insert DNA could have inserted into the *rop* gene in 2 orientations, resulting in a plasmid with two potential sequences. Even though the insert DNA was designed in a manner that ensures both orientations disrupt the *rop* gene, it is still essential to sequence this plasmid as the two different plasmids may produce different phenotypes.

The next step in testing the effect that the Rop protein has on the exclusion of pBR322 when co-transformed with pUC19 is to co-transform *E. coli* with pCAWK and pUC19 and compare the copy number of each plasmid. This can be achieved by performing restriction digest on the plasmids with a restriction endonuclease that would result in different sized fragments of the two plasmids for convenient identification (such as BsrFI). The intensities of the two plasmids and their resulting fragments can then be compared through gel-electrophoresis DNA staining to determine whether the copy number of pCAWK is higher than the copy number of pBR322 when both are separately co-transformed with pUC19. However, it is still possible that neither pCAWK nor pBR322 will be present in high enough concentration when co-transformed with pUC19 to be visible on a gel. The difference in plasmid size of pUC19 (2686 bp), and pBR322 (4361 bp) or pCAWK (4395 bp) provides an advantage for pUC19 in terms of resulting copy number as pUC19 will finish one replication cycle slightly faster than the two larger plasmids and over time, it will become dominant in the cell population (16). The second advantage of pUC19 is explained by the G to A point mutation in RNA II, reducing the binding affinity of RNA I, and therefore, allowing for increased replication (9). Therefore, it may also be desirable to co-transform *E. coli* cells with pCAWK and pBR322 and compare the Eco52I/EagI digested bands from the isolated plasmids directly on the gel to assess copy number. This ensures that the interpretation of results is not confounded by the other two copy number advantages that pUC19 possess.

If it is observed that pCAWK is present in higher copy number than pBR322 with either co-transformation proposed above, it supports the importance of the *rop* gene and its role in the exclusion effect of pBR322 when co-transformed with pUC19. The continued replication of both plasmids in one cell may be valuable as both plasmids have unique recognition sites and this allows for the incorporation of different gene products on each plasmid, which can then be co-expressed. Further experiments are required to fully characterize pCAWK and assess its value in molecular biology research.

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