Attempt to Construct a $\text{Rop}^+$ pUC19 from pBR322 Mutant lacking the Tetracycline Resistance Gene

DELIA WANG
Department of Microbiology and Immunology, UBC

The two commonly used plasmid vectors, pBR322 and pUC19, are both derivatives of the parent plasmid, pColE-1. However, it has been observed that pBR322 is excluded from the host cells following co-transformation of pBR322 and pUC19. One potential explanation for this exclusion effect is the presence of a functional $\text{rop}$ gene in pBR322. The $\text{rop}$ gene encodes for a protein that stabilizes the interaction between RNA I and RNA II, thus inhibiting the RNA II from carrying out its function as a primer to begin DNA replication. It is hypothesized that the lack of a functional $\text{rop}$ gene in pUC19 accounts for its dominance in the co-transformation with pBR322. An attempt was made to isolate a fragment containing the $\text{rop}$ gene from the tet$^+$ pBR322 mutant, and inserting it into pUC19 to create a $\text{rop}^+$ pUC19 construct. The $\text{rop}$ gene was successfully excised using restriction endonucleases AccI and EcoRI. However, ligation and subsequent transformation produced no viable clones. Due to time constraints, ligation and transformation conditions could not be optimized, but a number of different options are discussed in this study. The ultimate success of creating a $\text{rop}^+$ pUC19 will allow comparisons of co-transformation to be performed using pBR322 and either the wildtype or mutant $\text{rop}^+$ pUC19. Insights will be gained about whether the $\text{rop}$ gene contributes significantly to the exclusion effect.

Plasmid pUC19 and pBR322 are multipurpose cloning vectors both derived from the ColE1 plasmid (7). The replication control mechanism of pUC19 and pBR322, thus, is identical to that of ColE1 and its relatives (6). The typical replication process of ColE1-type plasmids involves a precursor primer, the RNA II transcript, hybridizing to a DNA sequence near the replication origin (6). RNase H cleaves bound RNA II, which then activates the primer to begin DNA synthesis. A RNA I transcript that is anti-sense to the RNA II is also made (6). When RNA I:RNA II hybridization occurs, the resulting conformational change inhibits RNA II from binding to its template DNA (6). Since the primer is absent, and RNase H only cleaves bound RNA II, DNA synthesis is suppressed (6). Although pUC19 is originally derived from pBR322, when both are co-transformed into a host cell, pUC19 has been observed to have a higher copy number than pBR322. Moreover, an exclusion effect is seen in which pBR322 is selectively eliminated from the host cell (7).

Several factors may contribute to this phenotypic difference. The size difference of the two plasmids is first taken into consideration. The pBR322 plasmid (4361bp) is about twice the size of pUC19 (2686bp), thus is likely to have a slower replication rate (9). Since the frequency of replication of the larger plasmid, pBR322, is also much lower than pUC19, pBR322 may eventually eliminated as host cells replicate (9).

The second factor postulated to lead to the exclusion effect is the presence of a point mutation in the coding region of RNA II of pUC19 (1). This $\text{G} \rightarrow \text{A}$ mutation located in nucleotide 112 of RNA II results in decreased RNA I:RNA II binding efficiency compared to pBR322 (1). Since more RNA II is allowed to freely bind its DNA template, more copies are replicated (1).

The presence of a $\text{rop}$ gene in pBR322 and the absence of it in pUC19 may largely influence differences in replication control mechanism. The $\text{rop}$ protein also plays a part in controlling the replication mechanism of ColE1-type plasmids. It is known that this 63 amino acid protein enhances the initial pairing between RNA I and RNA II transcripts, therefore further prevents the binding and activation of the RNA II primer for replication (6). In other words, a functional $\text{rop}$ protein negatively regulates plasmid copy number through an anti-sense control mechanism, such as seen in pBR322 but not pUC19 (8). As with any protein in cells, an overexpressed protein causes physiological stress and toxic damages to the cells (2). This is a concern with high copy number plasmids such as pUC19 (9).

This study aims to determine whether the presence of a $\text{rop}$ gene in pBR322 is responsible for the exclusion effect of pBR322 when co-transformed with pUC19 into E. coli DH5α. An attempt was made to construct a $\text{rop}^+$ pUC19 plasmid in hopes to ultimately co-transform it with pBR322 to compare with the original observation of the exclusion effect when pUC19 and pBR322 were co-transformed. It is known that pUC19 possess partial $\text{rop}$ gene sequence, but it lacks the ribosome binding site and the first 157 bp of the coding region (4). In pBR322, there has not been a
promoter found that is directly upstream of the rop gene (4). Previous work suggests that the rop gene is transcribed by a readthrough through the tet gene (3). Therefore, Ng (4) determined it essential that the tet gene P2 promoter and the –10 and –35 regions of rop are moved together with the rop gene (4). Previously, pBR322 mutants (pIN051-pIN0530) that lack the tetracycline resistance genes were created (4). The removal of the tetracycline resistance gene in pBR322 ensures that when rop gene is excised along with the tet gene P2 promoter region, tet coding region is not transferred into pUC19 (4). This restriction on the insert makes it possible to attribute the effect of the new pUC19 construct to the rop gene only (4). The tet pIN051 and pIN055 were confirmed for loss of tetracycline resistance. The mutant clones also possess a potential fragment that encloses the rop gene by two different, unique restriction sites, AccI and EcoRI.

This experiment was designed to isolate the rop gene from the tet pBR322 plasmids by restriction endonuclease digestion, and subsequently ligate the rop into pUC19 plasmid to assess the effect of rop on relative stability. The resultant rop pUC19 is hypothesized to significantly decrease in copy number. In a co-transformation experiment of rop pUC19 and pBR322 into E.coli DH5α, it is expected that instead of the selective exclusion of pBR322, a different observation will be made. This investigation would allow more advances in understanding the replication control mechanism in ColE1-type plasmids and ultimately create improved multi-purpose cloning vectors.

MATERIALS AND METHODS

Screening of mutant clones. Two mutant pBR322 clones (4), pIN051 and pIN055, were screened by testing for loss of tetracycline resistance. Colonies from plated cells were streaked onto Luria Bertani (LB) (5) plates containing either ampicillin (100 μg/mL) or ampicillin (100 μg/mL) and tetracycline (25 μg/mL). The plate cultures were grown at 37°C overnight.

Isolation of plasmid DNA. Culture tubes with LB broth (5) and 100 μg/mL ampicillin were inoculated with E.coli DH5α containing either pBR322, pIN051/pIN055, or pUC19, and grown overnight at 37°C with shaking at 200 rpm. The pBR322, pIN051, and pIN055 plasmid DNA were isolated using the Invitrogen PureLink™ HQ Mini Plasmid Purification Kit (Cat.# K2100-01). The protocol for low-copy plasmids was followed. The pUC19 plasmid was isolated using GenJet™ Plasmid Miniprep Kit from Fermentas (Cat.# K0501). The protocol for high-copy plasmids was followed in the handbook provided with the kit.

A modified alkaline lysis method was also used to isolate DNA (5). Two tubes with 10 ml of LB broth (5) containing 24 μg/mL ampicillin each were inoculated with a loopful of strain IN051 or strain IN055. Samples were centrifuged in Biofuge 17R (Program 2108, rotor 2147) at 4,000 rpm for 10 minutes at 4°C. The resulting pellets were washed with 500 μL STE buffer (5) per tube. The mixtures were vortexed, then centrifuged at 4,000 rpm for 10 minutes at 4°C. The pellet was resuspended in 200 μL of Solution I (5) per tube and vortexed briefly. Next, 400 μL of Solution II (5) were added to each tube and mixed by gently inverting five times. The mixture was immediately stored on ice, then 300 μL of ice cold Solution III (5) was added to each tube. This was followed by gentle mixing by inversion and incubation on ice for 3 to 5 minutes. After centrifuging at 4,000 rpm for 15 minutes at 4°C, the supernatant was transferred to fresh microcentrifuge tubes by pipetting. An equal volume of 1:1 phenol-chloroform was added and vortexed. Then, 600 μL was transferred to a new tube and precipitated the DNA by adding 600 μL of isopropanol at room temperature. The mixture was then mixed by vortexing and left at room temperature for 2 minutes. At the final step, pellets of the same sample were combined and redissolved in 200 μL of TE containing DNase-free pancreatic RNase (20 μg/mL). Dialysis tubings (Cat#25-131156) were boiled in 10 mM EDTA for 10 minutes. Samples were dialyzed at 4°C overnight, gently stirred, in Tris buffer (10 mM, pH 7.4) with 100 μL chlorofoam added.

Plasmid digest conditions. All plasmids were digested with AccI (NEBiolabs Inc., Cat.# R01615) and EcoRI (NEBiolabs Inc., Cat.# R01015). Samples with 10 μg of pBR322 were digested using 31.3 U of AccI, 34.8 U of EcoRI, and 1X NEBuffer 4. Samples with 10 μg of pIN051 or pIN055 were digested using 31.3 U of AccI, 17.4 U of EcoRI, and 1X NEBuffer 4. Samples with 10 μg of pUC19 were digested using 0.93 U of AccI, 0.53 U of EcoRI, and 1X NEBuffer 4. All reactions were made up to the final volume of either 20 μL or 30 μL with sterilized distilled water. All digest reactions were incubated at 37°C for 2 hours, then heat-inactivated at 65°C for 20 minutes.

Recovery of agarose gel bands. DNA Extraction Kit from Fermentas (Cat.# K0513) was used to recover desired DNA fragments from 0.8% Agarose II, low melting temperature agarose (MIDSCI, Cat.# 0815). The procedures followed the protocol provided with the kit.

Ligation of rop gene into pUC19. To ligate the rop gene fragment into the digested pUC19 vector, a ligation mixture with the following components was made up: 1 μg digested pUC19, 1 μg digested tet pBR322 fragment containing the rop gene, 12 U of T4 DNA Ligase (Fermentas, Cat.# EL0011), and 1X DNA Ligase Reaction Buffer (Fermentas). The reaction was made up to a final volume of 16 μL with sterilized, distilled water. After a brief vortex and centrifugation in a tabletop centrifuge for 3 to 5 seconds, the reaction was incubated at 22°C for 1 hour. This is followed by inactivation of the ligase by heating at 65°C for 10 minutes. All 5 ligation reactions were pooled and stored at –20°C until use.

Transformation of competent cells by chemical method. E.coli DH5α chemocompetent cells were previously prepared (4). Transformation was done by first adding 2 μL of ligation product to 200 μL of chemocompetent cells. This mixture was incubated on ice for 10 minutes, heat shocked at 42°C for 30 seconds, and inactivated on ice for 10 minutes. Then, 1 μL of LB broth, pre-warmed to 37°C, was added to the cells without any pipetting. This mixture was incubated for 1 hour at 37°C, shaking at 200 rpm. Finally, the culture was plated at various volumes ranging from 0.5 μL to 200 μL onto LB plates containing 100 mg/mL ampicillin. The surface of the LB plates were spread with 40 μL of 40 mg/mL X-gal and incubated for 30 minutes prior to plating transformation mixtures. pUC19 DNA (Invitrogen Cat.# 5437) was also attempted to be transformed into E.coli DH5α cells.

RESULTS

Identification of digested fragment from mutant tet pBR322 containing the rop gene. Figure 1 shows the restriction map of pBR322 with only unique restriction sites. The pBR322 plasmid contains AccI restriction sites at positions 651 and 2244 and an EcoRI restriction site at position 4359. The expected band sizes resulting from the double digestion are 2115 bp, 1593 bp, and 653 bp. The gel picture in Figure 2 shows that actual bands obtained were 2200 bp, 1600 bp, and...
In the *tet* intermediates, pIN051/pIN055, the AccI restriction site at position 651 is absent. Therefore, both AccI and EcoRI cleave at unique sites in pIN051/pIN055, and are expected to produce bands 2115 bp and 1014 bp in size. From the gel run, fragments with sizes 2200 bp and 800 bp were produced (Fig. 2). The 800 fragment is expected to contain the *rop* gene and was subsequently extracted from the gel. Digestion of the pBR322 plasmid ensured that the restriction endonucleases only cut at the correct sites.

![FIG. 1. Restriction map of the pBR322 plasmid. Digestion of this plasmid with restriction enzyme AccI forms cuts at position 651 and 2244. EcoRI cuts at position 4359. Therefore, three bands are expected as a result of a double digestion, which have sizes of 2115 bp, 1593 bp, and 653 bp. The *tet* intermediates (3129 bp), pIN051/pIN055, were originally cleaved by AfeI at position 496 and 1728 and recircularized. They form two bands from restriction digests by AccI at position 2244 and by EcoRI at 4359. The resulting band sizes are expected to be 2115 bp and 1014 bp. Theoretically, the 1014 bp fragment would possess the segment of the *tet* intermediate that contains the *rop* gene.](image)

Using the ligation product that remained after bacterial transformation, an analysis by gel electrophoresis was performed to confirm its identity. The *rop* gene insert was expected to ligate site-specifically in the MCS of the pUC19 plasmid (Fig 3) and add 800 bp to this 2686 bp *tet*-pBR322 mutant. Therefore, the expected size of the band produced should be around 3486 bp. However, the gel analysis produced a fragment of 2000 bp (Fig. 4). When the 2000 bp fragment was compared to the digested pUC19 DNA, no significant difference in sizes was observed.

![FIG. 2. Gel electrophoresis showing the result of a double digestion using AccI and EcoRI. A 0.8% low melting point agarose gel was used, run at 105V for 1 hr. Lane 1 shows the MassRuler™ Express DNA Ladder (Fermentas). Lane 2 shows bands at 2200 bp, 1600 bp, and 650 bp as the result of the double digestion of pBR322 digested as a positive control. Lanes 3 and 4 are IN051 and IN055, respectively. Both show bands at 2200 bp and 800 bp as a result of double digestion using AccI and EcoRI. The 800 bp is assumed to contain the *rop* gene.](image)

**Ligation of *rop* gene fragment into pUC19 vector.**
Transformation of chemically competent *E. coli* DH5α cells with 120 ng of pooled DNA from the ligation product resulted in no colonies on LB plates with ampicillin and X-gal. It was expected that the successful transformants would have a disrupted alpha complementation, and therefore form white colonies in the presence of X-gal. Various volumes of ligation product were plated, ranging from 0.5 μL to 200 μL, in hopes that a larger volume plates increase chances of obtaining viable colonies. Transformation was also attempted using commercially available pUC19 DNA.

However, none of these efforts produced any colonies on LB plates with ampicillin and X-gal.

**DISCUSSION**

From the pBR322 mutants lacking the tetracycline resistance genes, pIN051/pIN055, attempts to excise out a fragment containing the *rop* gene were made using restriction endonucleases AccI and EcoRI. Previous work done by Ng (4) suggested that the majority of the tetracycline gene was removed by cutting with AfeI at position 496 and 1728. The recircularized mutant was 3129 bp, consistent with Ng’s choice of re-ligating the 3000 bp fragment extracted from the gel. Based on this information, two
FIG. 3. Restriction map of the multiple cloning site (MCS) of the pUC19 plasmid. Digestion of the pUC19 plasmid with AccI and EcoRI should result in unique cuts in the MCS of the plasmid, positioned at 431 and 396, respectively. Through manual matching of the bases, it is expected that the 1014 bp insert cleaved from IN051/IN055 should ligate into the MCS in-frame. This insert includes a tetracycline P2 promoter, and is expected to transcribe in opposite direction of the pUC19 replication.

FIG. 4. Gel analysis of the digested pUC19 DNA and the ligation product to confirm the identity of the ligation product. Lane 2 shows the ligation product at 2000 bp. Lane 3 shows the digested pUC19 DNA also at 2000 bp. Lane 1 is the MassRuler™ Express DNA Ladder. A 1% agarose gel was used, run at 106 V for 50 min.

unique restriction sites that enclose the rop gene were located: EcoRI at position 4359, and AccI (or XmiI) at position 2244. Cleavage of the pBR322 plasmid using these two restriction enzymes produced three expected band sizes since the wildtype contains two AccI cut sites. However, the fragment expected to contain the rop gene resulting from cleavage of the tet mutants was at slightly smaller size, 800 bp, instead of the expected 1014 bp. Restriction digests by the same enzymes were performed multiple times and yielded the same result in a gel run. This could be explained by possible cleavage of a longer fragment than expected during the removal of the tetracycline resistance gene. Although AfeI restriction sites have been manually matched to the sequence of pBR322, the exact sequences of pIN051/pIN055 have not yet been determined.

There are several factors that possibly explain to the absence of the expected 3486 bp band in the gel electrophoresis of the ligation product. One explanation traces back to the possible failure of ligation. Due to time constraints, the ligation attempt was tried only once with 1 μg of the rop gene insert and 1 μg of digested pUC19 vector. In addition, no purification step was carried out to any of the DNA due to the risk of losing DNA content particularly when working with such small volumes. This limitation could also decrease the amount of the DNA of interest in both the rop gene insert and the pUC19 vector. In other words, the actual volumes of the insert and vector added were likely less than volumes that were measured. Of the many insert-vector ligation product combinations that are possible, the chances of getting the circularized rop gene ligated to pUC19 is less than 1 in 6. These combinations include self-ligation of the insert, self-ligation of the vector, linearized vector-insert, circularized vector-insert, and vector or the insert alone. Since the amount of DNA used for ligation was very little, the resulting bands on the gel are expected to be very faint since DNA material was divided among fragments formed from all the possible combinations. Upon more careful comparison of gel band intensities, the 2000 bp band in lane 2 (Fig. 4) is estimated to have less than 20 ng of DNA. This band indicates the presence of a linearized pUC19 vector without any insert. Since equal volumes of insert and DNA were ligated, it was expected that another band of the rop gene insert should be observed at the 800 bp position with similar intensity as the pUC19 vector. The absence of a 800 bp band means that some rop gene insert DNA must have been lost in the process of transferring and mixing of reaction mixtures. However, the lack of bands does not suggest definitive failure of ligation. The bands may be sufficiently faint that they were invisible to the detector.

The gel run of the ligation product produced only one fragment (2000 bp) (Fig. 4). This band matched with the size of digested, linear pUC19, and would also explain the lack of viable colonies when ligation products are plated. If the plasmid failed to
recircularize, it leads to a nonviable product that loses the ability to provide ampicillin resistance to the host *E. coli* cells (4). In the case that the host cell possesses a circularized plasmid of two inserts ligated to each other, there is no origin of replication nor the ampicillin resistance gene to protect the host cell in an environment with ampicillin. The chances of obtaining a re-circularized vector or vector-insert plasmid that will provide ampicillin resistance to the host cell are slim. Therefore, a large volume of transformants should be plated to observe any colony growth. However, when commercially available pUC19 was transformed and plated at the same volumes on LB plates with X-gal and ampicillin, there was still no colony growth. Therefore, it is also likely that the batch of chemically competent *E. coli* DH5α cells were incorrectly handled or were not prepared properly to cause their incompetency in taking up plasmids and forming viable colonies.

**FUTURE EXPERIMENTS**

To take this investigation on the role of the *rop* gene forward, there are several modifications that could be performed in constructing a *rop*+ pUC19. After cloning the *tet* pBR322 mutant, the first confirmation to be made is to sequence its entire plasmid DNA. Since the fragment containing the *rop* gene is shorter than expected by 200 bp, this will check whether the *Afe*I excisions were actually made at position 496 and 1728. Another option to overcome this problem is to further cut the *tet*-intermediate at unique restriction sites located within 200 bp before the *Afe*I site at 496 and after *Afe*I site at 1728. Examples of these unique restriction sites are *Tst*I at position 389 and *Nhe*I at position 229. No cleavage should be observed if the *Afe*I cuts were originally made at the correct positions, but the presence of additional bands indicates that a larger fragment than expected was removed.

While conditions required for optimized ligation reaction are not yet well understood, more attempts of varying conditions would provide additional insights. Moreover, it would allow higher accuracy in measuring the actual working volumes of DNA. It should also be considered to use a higher ratio of insert to vector as this may affect the efficiency of ligation. It is suspected that the batch of chemically competent *E. coli* cells may not have been properly handled during its production and usage. Even when commercially available pUC19 was attempted to be transformed into these chemically competent cells, no colonies were produced. The first attempt to solve this problem should be to prepare fresh chemocompetent *E. coli* DH5α cells using the same procedure, but take extra care in handling the cells. Commercially available competent *E. coli* DH5α could also be used. Subsequent transformation procedure should be repeated using the fresh batch of chemocompetent cells with careful handling of the transformation mixture. Since there are several ways in which digested fragments could ligate, increasing the efficiency at which the desired ligation product will form is important to the success of transformation. One way to prevent vector self-ligation is by treating the pUC19 with alkaline phosphatase. This will remove the 5′ phosphates and promote the untreated *rop* gene insert to subclone into the treated pUC19 (4). Finally, results from co-transformation of *rop*+ pUC19 and pBR322 can be compared to co-transformation of *rop* pUC19 and pBR322 to observe for changes in exclusion and plasmid copy number. This will answer the question of whether the *rop* gene plays a role in the exclusion effect.

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