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The Construction of pBR322Δ*rop* and Its Interaction with pUC19 with Respect to Plasmid Copy Number and the Exclusion Effect

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When pBR322 is co-transfected with pUC19, pBR322 is excluded from the host bacterial cell in what is known as the exclusion effect. The pBR322 plasmid encodes the *rop* gene that regulates copy number, which pUC19 lacks and thus has a high copy number. The *rop* gene was removed from pBR322, to create pBR322Δ*rop*, to investigate whether Rop is responsible for a decreased plasmid copy number and is thus the cause of the exclusion effect. Co-transfections of pUC19 with either pBR322 or pBR322Δ*rop* were performed in *Escherichia coli* DH5α to determine if pBR322Δ*rop* would be able to evade the exclusion effect. Through selection with antibiotics and blue and white colony screening, it was found that when pUC19 was co-transfected with pBR322Δ*rop*, the pBR322 derivative had evaded the exclusion effect as there was a greater frequency of white colonies, which contained only pBR322Δ*rop*. However, there were seven times more white colonies than blue colonies, suggesting the absence of pUC19 in most of the colonies. This implies that there is a possibility that pUC19 is excluded out of the *E. coli* when co-transfected with pBR322Δ*rop*. Nonetheless, since such a small population was analyzed in this experiment, because of time constraints, further experiments should be performed to confirm the possible exclusion effect of pUC19 by pBR322Δ*rop*.

Plasmids that are able to co-exist in a host allows for the study of the necessity of certain gene products or their interaction with one another in a positive or negative manner. In the case of pBR322 and its derivative, pUC19, there are two factors that may play a role in the regulation of the interaction of these two plasmids.

The regulation of plasmid replication in ColE1-type replicons involves the interaction between two plasmid-specific RNA transcripts, RNAI and RNAII (1). Initiation of replication begins with RNAII, where it functions as a primer for DNA synthesis as it forms a persistent hybrid with the DNA template near the replication origin (1). However, two events prevent primer formation. Firstly, RNAI is complementary to a section of RNAII and these two transcripts can bind together in an antisense manner to form an unstable stem-loop structure complex that inhibits RNAII from binding the DNA template (3). Secondly, the Rop protein, encoded by ColE1-type replicons, also aids in RNAI/RNAII complex stability and thus regulates plasmid replication further (1). The plasmid pBR322 is regulated by this RNAI/RNAII/Rop protein interaction and thus is a low copy plasmid.

The plasmid pUC19 is derived from pBR322 but replicates at high copy number due to a single point mutation in RNAII (4) and moreover, this mutation can not be stabilized by the Rop protein, which pUC19 does not encode. Because of the instability of the RNAI/RNAII complex caused by the absence of the *rop* gene and the presence of the RNAII point mutation, pUC19 is able to initiate replication frequently.

In previous Microbiology 321 and 421 classes (UBC), it was noticed that when pBR322 is co-transfected in equal amounts with pUC19, pBR322 is excluded from the host. Plasmid incompatibility, similar to the exclusion effect, was observed by Nugent *et al.* (5) with pBR322 and a pBR322-based plasmid, pWT111. The removal of the *rop* gene from pWT111 resulted in a copy number increase and it was discovered that the Rop protein was involved in the strong plasmid incompatibility observed between pWT111 and pBR322. Thus, based on the Lin-Chao *et al.* (4) hypothesis, the observation of pUC19 exclusion of pBR322 may be due to Rop activity in pBR322. To test this explanation, the *rop* gene was removed from pBR322 to create pBR322Δ*rop* to determine whether the Rop protein is vital to the exclusion effect of pBR322.

MATERIALS AND METHODS

Cultures and plasmids. Cultures of *Escherichia coli* HB101 (W. Ramey, UBC) containing the plasmids pBR322 and pUC19, respectively, were grown in Luria-Bertani (LB) broth to an OD_{660} of approximately 0.7, at that time chloramphenicol (0.28μg/mL) was added to amplify the plasmids and inhibit growth as the cultures were incubated overnight. The plasmids were then isolated and purified using the Gibco BRL® Concert™ High Purity Plasmid Maxiprep System according to the manufacturer's protocol. The purified pBR322 and pUC19 were resuspended in Tris-EDTA buffer and distilled water, respectively.

Digestions and ligations. Initially, pBR322 was digested with *Pvu*II (Gibco BRL) and *Msc*I (Invitrogen), to unsuccessfully remove the *rop* gene. An alternate overnight digestion was performed with *Msc*I and *Nde*I (Invitrogen) in which the sticky ends were subsequently filled-in with DNA polymerase I Klenow fragment (Invitrogen) to generate blunt ends. The sample was then run on a 1% agarose TBE gel, along with pBR322 linearized with *Nde*I, undigested pBR322, and 1kb Plus DNA Ladder (Invitrogen) as a standard. The fragment representing pBR322Δ*rop* at 3511bp was excised using the QIAEX II Agarose Gel Extraction Kit (Qiagen) according to the manufacturer's protocol, in which the DNA was eluted with distilled water (pH 8.5). The ends of the linearized pBR322Δ*rop* DNA were then ligated together using T4 DNA ligase (Invitrogen). From the ligation mixture, the enzymes were removed and the ligated pBR322Δ*rop* was concentrated using the QIAEX II Agarose Gel Extraction Kit (Qiagen) according to the manufacturer's Desalting and Concentrating DNA Solutions protocol, in which the pBR322Δ*rop* DNA was eluted with distilled water (pH 8.5).

Transformations. *E. coli* DH5α cells (W. Ramey, UBC), an alpha-complementing strain which unknowingly contained pBR322, were grown to an OD_{600} of 0.2 at which time the cells were rendered competent according to the Zero Background™/Kan Cloning Kit (Invitrogen) protocol. However, only half of the volumes stated in the protocol were used. A sample of the competent *E. coli* DH5α cells was transformed with 2.5μg of pBR322Δ*rop* utilizing the Bio-Rad MicroPulser™ Electroporation Apparatus according to the Bio-Rad MicroPulser™ manual. A positive control using 1μg pBR322 and a negative control not containing plasmid, were also transformed. A 0.2mm cuvette and the pulse setting Ec2 were used for each sample. The electroporated cells were then resuspended in LB broth for a recovery period of 1.5 hours after which 200μL of each culture was spread onto an Ampicillin (50μg/mL)/LB plate. The resulting colonies were used as inoculum and grown overnight in an LB/Ampicillin broth. The cultures were then pelleted at top speed for 2 minutes in the Heraeus Instruments microfuge. The plasmids were then isolated and purified using the Gibco BRL® Concert™ High Purity Plasmid Miniprep System according to the manufacturer's protocol, in which the samples were resuspended in distilled water (pH 8.5). 3μL of each sample was digested with *Nde*I for 2 hours and then analyzed on a 1% agarose TBE gel, along with pBR322 linearized with *Nde*I and 1kb Plus DNA Ladder as a standard. Co-transformations of pUC19, an alpha complementing plasmid, and different forms of pBR322 were performed as in the previously stated electroporation method. There were four different combinations of co-transformations: 2μg each of pUC19 and pBR322, and pUC19 and pBR322Δ*rop* at concentrations of 2μg, 3μg and 5μg each. The electroporated cells were then resuspended in LB broth for an overnight recovery period after which 100μL of each culture was spread onto an Ampicillin/LB plate, with 0.8mg each of X-Gal and IPTG, and incubated overnight. The resulting overnight cultures were too concentrated and were plated again onto Ampicillin/X-Gal/IPTG/LB plates at three different dilutions: 10^{-7}, 10^{-8}, and 10^{-9} cells. These plates were then replica plated onto Tetracycline (15 μg/mL)/LB plates with 0.8mg each of IPTG and X-Gal, and incubated overnight. The two sets of plates were then compared to determine whether pBR322Δ*rop* was excluded from the transformed DH5α *E. coli* cells.

RESULTS

The pBR322 plasmid, isolated using the Gibco BRL® Concert™ High Purity Plasmid Maxiprep System, was subjected to two restriction enzyme double digests with differing conditions in an attempt to remove the *rop* gene. The *Pvu*II /*Msc*I double digest first resulted in the star activity of *Pvu*II, where many fragments were generated (gel not shown) and secondly, resulted in the production of two fragments as shown in Figure 1.

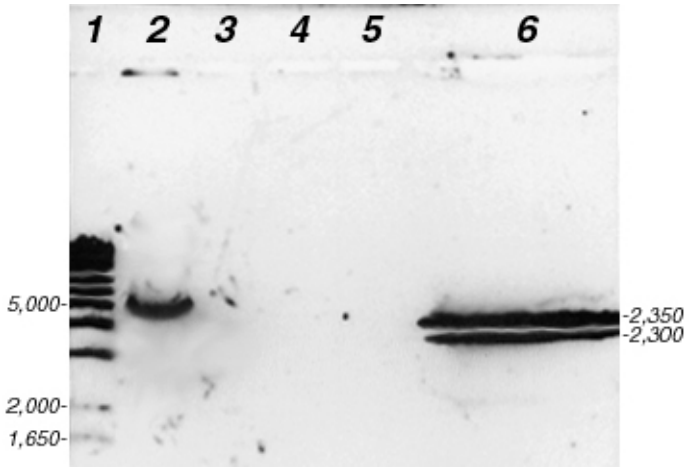

Figure 1. *Pvu*II /*Msc*I double digest of pBR322 in an attempt to remove the *rop* gene. The following samples were run on a 1% agarose gel: Lane 1 – 0.9μg 1kb Plus DNA Ladder; Lane 2 – 2.5μg uncut pBR322; Lanes 3-5 – empty; Lane 6 – 5μg cut pBR322

The production of two similarly sized bands in cut pBR322 suggested that the restriction sites, as stated by New England Biolabs, were not correct. When compared against the 1kb Plus DNA Ladder, the bands of the uncut pBR322 sample in Lane 6 of Figure 1 do not correspond to the sizes of the standard and was due to the uneven setting of the wells in the agarose gel. However it was deduced that a complete digest with *PvuII* of the uncut pBR322 was achieved. After consultation with GenBank, it was discovered that *Pvu*II cut not only at 2075bp, but also at 5bp, which resulted in the separation of pBR322 into two almost equal halves of 2300bp and 2350bp.

Thus an *Msc*I/*Nde*I double digest was performed since the restriction sites were approximately in the same location as the *Msc*I/*Pvu*II double digest, as shown in Figure 2.

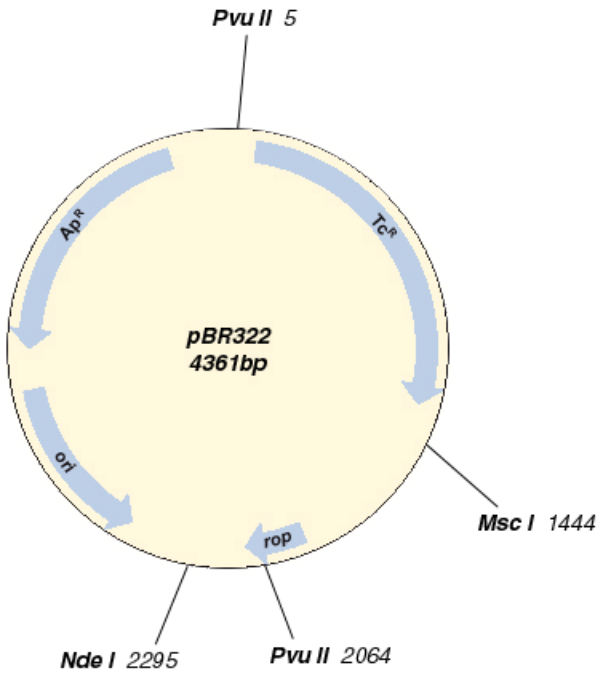

Figure 2. pBR322 restriction map with restriction sites relevant to this experiment.
The second restriction site of *Pvu*II at 5bp, as predicted from the pBR322 GenBank entry (www.ncbi.nlm.nih.gov), is shown on the modified restriction map as stated by New England BioLabs (www.neb.com).

An overnight *Msc*I/*Nde*I digestion was performed and resulted in an incomplete digest as shown in Figure 3. However, the uncut pBR322 and pBR322Δ*rop* bands were separate enough from each other so that the pBR322Δ*rop* band was extracted for further use. Uncut pBR322 and linearized pBR322 were run as standards to ensure that the band representing pBR322Δ*rop* was not super-coiled pBR322, which runs at a lower molecular weight.

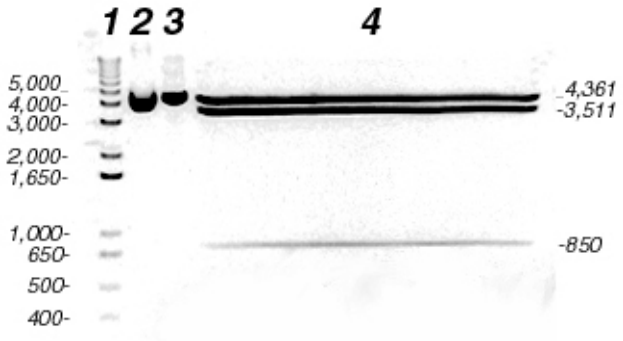

Figure 3. *Msc*I/*Nde*I double digest of pBR322 to remove the *rop* gene.
The following samples were run on a 1% agarose gel: Lane 1 – 0.9µg 1kb Plus DNA Ladder; Lane 2 – 2.5µg uncut pBR322; Lanes 3 – 2.5 µg pBR322 linearized with *Msc*I; Lane 4 – 5µg cut pBR322, where 4361bp represents uncut pBR322, 3511bp is pBR322Δ*rop* and 850bp is the *rop* gene.

Following gel extraction, the pBR322Δ*rop* band was then purified, treated with DNA polymerase I Klenow fragment for the generation of blunt ends, treated with T4 DNA ligase, desalted, and concentrated. The pBR322Δ*rop* was then transfected into DH5α *E. coli* cells and plated onto Ampicillin plates. The positive control transfected with pBR322 produced too many colonies to count while the negative control transfected without plasmid DNA produced three colonies. Six colonies were produced by pBR322Δ*rop* Sample 1 and three colonies were produced by pBR322Δ*rop* Sample 2. Each colony from the negative control and the two pBR322Δ*rop* samples were then grown overnight in an LB/Ampicillin broth overnight and then subjected to plasmid isolation. An aliquot of the plasmids were then linearized and run on a gel to verify the presence of pBR322Δ*rop* as shown in Figure 4. Linearized pBR322 was run as a standard to ensure that the isolated plasmids pBR322Δ*rop* were different from pBR322 in size.

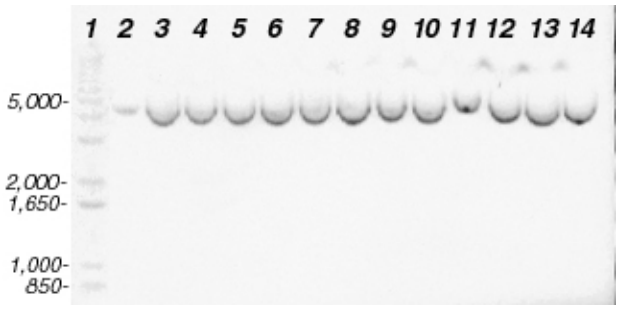

Figure 4. *Nde*I digestion of the isolated plasmids from the two pBR322Δ*rop* colonies. An aliquot of each isolated plasmid sample was linearized through a 2 hour *Nde*I digest and run on a 1% agarose gel: Lane 1 – 0.9µg 1kb Plus DNA Ladder; Lane 2 – 2.5µg linearized pBR322; Lanes 3-8 - pBR322Δ*rop* Sample 1 colonies; Lanes 9-11 – negative control colonies; Lanes 12-14 - pBR322Δ*rop* Sample 2 colonies.

Since the negative control colonies produced a band, which is the approximate size of pBR322, the host DH5α *E. coli* that was used in the transfection may have already contained pBR322. However, the plasmids isolated in the pBR322Δ*rop* samples produced a 3511bp band that verified the presence of pBR322Δ*rop* and the plasmid isolated from colony 2 of pBR322Δ*rop* Sample 2 was used in the remainder of this experiment.

Four co-transfection experiments were performed in DH5α *E. coli* as follows: 2µg pBR322, 2µg pBR322Δ*rop*, 3µg pBR322Δ*rop*, and 5µg pBR322Δ*rop* respectively with equimolar amounts of pUC19. The transfected cells were incubated overnight and then plated onto Ampicillin/IPTG/X-Gal/LB plates. X-Gal, a chromogenic substrate, was used to identify the presence of alpha-complementation between the β-galactosidase subunits produced by the pUC19 plasmid and the DH5α *E. coli* host, which would result in a Lac^+ bacteria that forms blue colonies. The result was a smear of colonies and thus the cells were plated at too high of a concentration. However, there was a the colour difference between the cells in which the positive control containing pBR322 and pUC19 was a dark turquoise blue while the cells transfected with pBR322Δ*rop* and pUC19 were a greenish colour, a mixture of yellow and blue colonies. Thus, the cultures were plated again, but at the dilutions of $2x10^{-7}$ and $2x10^{-8}$ cells. Each dilution was plated onto Ampicillin/IPTG/X-Gal/LB plates and then replica plated onto Tetracycline/IPTG/X-Gal/LB plates the following day. The results of the selective screening are shown in Table 1.

Table 1. Selective screening of pUC19 and pBR322 in DH5α *Escherichia coli*

Transfection Plasmid DNA[b]		2×10^{-7} Cells Dilution[c]				2×10^{-8} Cells Dilution			
		pBR322	pBR322Δ*rop*			pBR322	pBR322Δ*rop*		
		2µg	2µg	3µg	5µg	2µg	2µg	3µg	5µg
Ampicillin[a] (50µg/mL)	**Blue**	61	3	2	1	8	2	0	0
	White	3	22	14	11	0	2	1	1
Tetracycline (15µg/mL)	**Blue**	39	2	0	0	5	0	0	0
	White	3	21	10	9	0	2	0	0

[a] 0.8mg each of IPTG and chromogenic substrate X-Gal were spread onto Ampicillin and Tetracycline plates
[b] All samples were co-transfected with an equimolar amount to the stated pBR322/pBR322Δ*rop* DNA used
[c] All dilutions were spread onto Ampicillin plates and then replica plated onto Tetracycline plates.

On the Ampicillin plate, a white colony indicates that only pBR322 or pBR322Δ*rop* is present within the bacterial cell because pBR322 and its derivatives do not contain a β-galactosidase subunit within its genome and therefore does not have the ability to use the X-Gal chromogenic substrate along with the DH5α *E. coli* host. On the other hand, a blue colony indicates pUC19 alone, or pUC19 and either pBR322 or pBR322Δ*rop* are present. On the Tetracycline plate, a white colony indicates the presence of pBR322 or pBR322Δ*rop* and a blue colony indicates the presence of pUC19 and either pBR322 or pBR322Δ*rop*. As shown in Table 2, the positive control of pUC19/pBR322 produced more blue than white colonies, as expected because of the exclusion effect, while the experimental samples transfected with pUC19 and pBR322Δ*rop* DNA produced more white colonies than blue.

DISCUSSION

When pBR322 was co-transfected with pUC19 in this experiment, the majority of the colonies were blue in both dilutions, suggesting that pBR322 had been completely excluded from approximately 30% of the colonies, as tabulated from Table 1. By analyzing the data collected from the selective screening of the colonies, a lesser dilution may have been more useful in giving a greater population for analysis than the set of 2 x 10^{-8} cells dilution plates.

An increased amount of white colonies were present in the experimental plates, when compared to the control plate, and indicates that pBR322Δ*rop* does evade the exclusion effect. The absence of the *rop* gene does play an important role in the increased frequency of the pBR322 derivative, pBR322Δ*rop*, when co-transfected with pUC19. These findings are concurrent with Nugent *et al.* (5) in which they removed the coding region of *rop* from the pBR322 copy number mutant, pWT111 to achieve an additional six-fold increase in copy number.

However, in each of the samples co-transfected with both pUC19 and pBR322Δ*rop*, there were greater than seven times more white colonies than blue colonies in the Ampicillin set of 2 x 10^{-7} cells dilution plates. When replica plated onto Tetracycline, less than 1% of the white colonies failed to grow and may have been due to mutation in which the bacterial cell was unable to pump out the Tetracycline molecules. The occurrence of more white colonies than blue colonies in the cells transfected with both pBR322Δ*rop* and pUC19 was not expected because it suggests the absence of pUC19 DNA within the cells. This implies that there is a possibility that pUC19 is excluded out of the bacterial host cell when co-transfected with pBR322Δ*rop*. Nonetheless, because of time constraints and since such a small population was analyzed in this experiment, further selective plating experiments should be performed to confirm the possible exclusion effect of pUC19 when co-transfected with pBR322Δ*rop*.

To further confirm that the removal of the *rop* gene from pBR322 does enable pBR322Δ*rop* to escape the exclusion effect, the approach of Lin-Chao *et al.* (4) should be investigated with further co-transfection experiments involving differing incubation temperatures with pBR322Δ*rop* and a constructed pUC19 plasmid that contains the *rop* gene. Firstly, this approach would verify that the *rop* gene is responsible for regulating copy number if a decrease in the copy number of pUC19 with *rop* occurs. Secondly, if the *rop* gene was inserted into pUC19 in such a way that insertional inactivation would not occur and was then co-transfected with pBR322Δ*rop*, the exclusion effect of pUC19 and its derivatives by pBR322Δ*rop* would be verified if there were still more white colonies than blue colonies.

As well, based on the findings by Lin-Chao *et al*. (4) that a single point mutation in RNAII of pUC19 was a factor in enabling an increase in copy number, an experiment should be performed where the exact single point mutation should be engineered into the pBR322 plasmid. This investigation would determine whether the single factor of either the point mutation or absence of the Rop protein is mainly responsible for an increase in copy number, or whether both factors are necessary for the exclusion factor. The results from this investigation can then be compared with the study by Nugent *et al*. (5) in which a point mutation in RNAI resulted in an eight-fold increase in copy number while the removal of *rop* from pWT111 resulted in a further six-fold increase in copy number.

Conversely, not only did pBR322Δ*rop* appear to escape the exclusion effect, it seemed to exclude pUC19. These two plasmids could not co-exist in a host and thus were not as equals as pBR322Δ*rop* was more dominant. If this observation was authentic, it may arise that pBR322Δ*rop* is more unregulated than pUC19 where the deletion of *rop* from pBR322 may allow less regulatory function than a point mutation in RNAII. If this is the case, an investigation should be conducted to determine whether a point mutation in RNAII in pUC19 is less efficient in plasmid replication than pUC19 with an added *rop gene* and non-mutated RNAII.

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