Journal of Experimental Microbiology and Immunology (JEMI)

Exclusion of pBR322 after co-transformation with pUC19 into *Escherichia coli* is mediated by the *rop* gene

Angela Mo, Thomas So, Wendy Zhang, Wouter-Jan van Roosmalen
Department of Microbiology and Immunology, University of British Columbia

Co-transformation of plasmids is often necessary and useful in molecular biology for purposes such as introducing multiple desirable characteristics into cells. It has been observed that co-transformation of pBR322 and pUC19, two commonly used vectors, into *Escherichia coli* results in the exclusion of pBR322, where cells that have taken up both plasmids lose pBR322 while retaining pUC19. The three characteristics of pUC19 that may contribute to its selective maintenance in the cell relative to pBR322 are (i) its smaller size, (ii) a G to A mutation in RNAII, and (iii) lack of a *rop* gene. We hypothesized that the presence of the *rop* gene on pBR322 results in its exclusion when co-transformed with pUC19, and the inactivation of *rop* would diminish this effect. The influence of the *rop* gene on plasmid exclusion was tested by using a derivative of pBR322, pCAWK, which contains a 34 bp sequence disrupting the *rop* gene. To measure the plasmid ratio in co-transformed cells, we developed a β-galactosidase white/blue colony ratio screening assay. When plasmids pCAWK and pUC19 were transformed into *E. coli* DH5α we observed a pCAWK:pUC19 ratio of 6.25. By comparison, when pBR322 and pUC19 were co-transformed into *E. coli* DH5α we observed only pUC19. This data suggests that pCAWK, which lacks the *rop* gene, can be maintained in the presence of pUC19 whereas pBR322 cannot. Taken together these data support the hypothesis that the *rop* gene on pBR322 contributes to its exclusion upon co-transformation with pUC19.

Plasmids are one of the driving elements of horizontal gene transfer and contribute to the plasticity of bacterial genomes. They can be used as cloning vectors to manipulate genes of interest or as expression vectors for protein production. One commonly used *Escherichia coli* cloning vector is pBR322.

Replication of pBR322 is initiated by the synthesis of RNAII, an RNA primer, followed by the binding of RNAII to the origin of replication (1). Plasmid replication can be negatively regulated by RNAI, which hybridizes with RNAII and prevents DNA binding (1). Negative regulation of replication is also mediated by the regulatory protein Rop, which accelerates and stabilizes the formation of the RNAI and RNAII complex (1). The overall effect of repressed pBR322 replication is a low plasmid copy number within the cell.

A derivative of pBR322 called pUC19 replicates to higher copy numbers than pBR322 (1). One reason for the higher level of replication is a G to A point mutation in the RNAII of pUC19 that affects the secondary structure of RNAII and prevents RNAI binding (1). In addition, pUC19 lacks the *rop* gene which decreases the stability of the RNAI and RNAII complex (2). It has also been proposed that the larger size of pBR322 may reduce the fitness of the host cell due to longer replication times (3,4). The metabolic burden of maintaining a large plasmid can only be relieved by losing the plasmid through segregation instability or by decreasing the plasmid copy number (3).

Co-transformation with different plasmids can be used to introduce multiple genes or regulatory elements into the same cell (5,6). However, both of the plasmids must also be maintained for co-transformation to be successful. It has been observed that when pBR322 and pUC19 are co-transformed into *E. coli* cells, pBR322 is selectively excluded (7). Cells that have taken up both plasmids lose pBR322 while retaining pUC19 (7). This phenomenon will be termed the "exclusion effect" for the purpose of this study and can be considered as the selective loss of one plasmid over another from the cell. The same three factors thought to contribute to higher pUC19 copy numbers (the G to A mutation, the lack of Rop, and the smaller plasmid size) have been proposed to contribute to the selective maintenance of pUC19 over pBR322 (4). Developing an understanding of the plasmid characteristics that contribute to the exclusion effect can ensure the success of future experiments involving co-transformation.

In this study we investigated the effects of the Rop protein by using pCAWK, a pBR322-derived plasmid containing a 34 bp insert that inactivates the *rop* gene (4). The insertional inactivation of *rop* is hypothesized to mitigate or reduce the exclusion effect when pCAWK is co-transformed with pUC19. Previous research on the topic of pBR322 exclusion have typically determined plasmid copy ratios by comparing the fluorescence intensity of the bands on a gel, which has poor sensitivity (8,9,10). We have developed a sensitive growth-based screening assay to measure the ratio of one plasmid to another in co-transformed cells. Our results show that the white/blue colony ratio from the assay can be correlated with the plasmid copy ratio in the cells. The assay was then used to determine that the Rop protein contributes to the selective loss of pBR322 when co-transformed with pUC19.

MATERIALS AND METHODS

Strains and plasmids. DH5α and *E. coli* strains containing the plasmids pBR322, pCAWK and pUC19 were obtained from the University of British Columbia (UBC) Department of Microbiology and Immunology strain collection.

Plasmid isolation. *E. coli* strains containing pBR322, pCAWK and pUC19 were grown as overnight cultures at 37°C in 5 mL of Luria Bertani (LB) containing 100 μg/mL of ampicillin. Plasmids were isolated from 2 mL of each culture using PureLink® Quick

Plasmid Miniprep Kits by Invitrogen and eluted in Tris-EDTA (TE) buffer. pCAWK was eluted in dH_2O for sequencing reactions. Plasmid concentration and purity were measured using the NanoDrop 2000c spectrophotometer.

Restriction enzyme digestion and agarose gel electrophoresis. pBR322 contains an EagI restriction site and pCAWK was constructed by inserting a sequence with another EagI restriction site into the *rop* gene of pBR322. Both of the plasmids were restriction enzyme digested with EagI for 16 hours. The digests were run against a Lambda DNA/HindIII marker and undigested pBR322, on a 1.2% agarose gel in TAE buffer at 80V for 1.5 hours followed by ethidium bromide staining for 20 min.

Sequencing of pCAWK. Primers flanking the insert in pCAWK were ordered from Integrated DNA Technologies. The forward primer sequence was 5'-AAC AAC ATG AAT GGT CTT CGG-3' and the reverse primer sequence was 5'-TAC AAT CTG CTC TGA TGC CG-3'. pCAWK eluted in dH_2O was sequenced at the Nucleic Acid Protein Sequencing Unit (UBC).

Preparation of competent cells. The Hancock Laboratory (UBC) $CaCl_2$ transformation protocol was used to make the DH5α cells competent (11). Briefly, a 1:200 dilution of a 5 mL overnight culture was grown in 50 mL of LB at 37°C to an OD_{550} of 0.2-0.4, then placed on ice for 10 min. The cells were centrifuged at 10,000 rpm and 4°C for 5 min in Oakridge tubes containing 20 mL each. After discarding the supernatant, 10 mL of 0.1 M $CaCl_2$ was used to resuspend the pellet. The cells were placed on ice for 20 min, then centrifuged again under the same conditions. This time, the pellet was resuspended in 0.4 mL of $CaCl_2$ and placed on ice for 1 hr. Aliquots of 100 µL were stored at -80°C in 15% v/v glycerol.

Co-transformations of *E. coli* DH5α with pBR322 and pUC19. Co-transformation was done by heat shock as outlined in the Hancock Laboratory (UBC) $CaCl_2$ transformation protocol (11). A 1:1 ratio of pBR322 and pUC19 was added at total plasmid amounts of 30 ng, 3 ng, 1 ng, 300 pg and 30 pg to aliquots of competent DH5α cells. The cells were then placed on ice for 20 min, heat shocked for a minute in a 42°C water bath, and placed on ice again for another minute. After adding 1 mL of LB, the tubes were incubated for an hour on a shaker at 37°C. LB agar plates containing 5 µg/mL of tetracycline were plated with 40 µL of 2% w/v X-Gal and 7 µL of 20% w/v IPTG, allowed to dry, then spread plated with 100 µL of the co-transformants. These co-transformations were performed to obtain pBR322/pUC19 double transformants and to determine the optimal plasmid amount at which double transformants is minimized. A standard curve was also generated by co-transforming pBR322 and pUC19 at varying molar ratios of pBR322 to pUC19 (9:1, 7:3, 1:1, 3:7 and 1:9). For each co-transformation, 3 ng of total plasmid was used, and the cells were plated on LB agar plates containing X-Gal, IPTG and 100 µg/mL of ampicillin.

White/blue screening and tetracycline selection to obtain pCAWK/pUC19 double transformants. pCAWK/pUC19 double transformants were obtained by first co-transforming pCAWK and pUC19 at a 1:1 ratio using 20 ng, 30 ng, 40 ng and 60 ng of total plasmid. The co-transformants were then plated on LB agar plates containing ampicillin, X-Gal and IPTG, and incubated at 37°C. Blue colonies were screened by first stabbing each colony into a LB-Tet plate, then a LB-Amp plate, and finally into LB-Amp liquid media. Both of the plates had X-Gal and IPTG, and a grid system was utilized to keep track of the cultures. Colonies exhibiting growth on LB-Tet and blue on LB-Amp indicated a double transformant and were selected for the white/blue colony ratio screening assay.

White/blue colony ratio screening assay. Plasmids were isolated from the pBR322/pUC19 and pCAWK/pUC19 double transformants using the method described previously. 3 ng of each isolate was then transformed into competent DH5α cells using the $CaCl_2$ transformation protocol also described previously, and plated on LB agar plates containing ampicillin, X-Gal and IPTG. The number of blue and white colonies was counted after incubation at 37°C overnight. An overview of the assay is visualized in Figure 1.

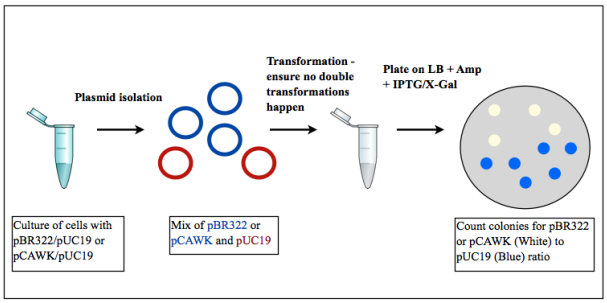

FIG 1 Workflow for white/blue colony ratio screening assay that quantifies the exclusion effect using white/blue colony counts. After ensuring that double transformations happen infrequently, each colony represents a transformant containing only one plasmid. The readout of white to blue colony ratios reflects the ratio of pBR322 or pCAWK to pUC19 harboured by the original double transformant.

RESULTS

Verification of pCAWK by restriction mapping using EagI and sequencing. The pCAWK plasmid sequence was first verified to contain the *rop* gene-inactivating insert in the correct location to ensure that it can be used to test the effect of the *rop* gene on plasmid exclusion. The 34 bp insert in pCAWK introduces a second EagI restriction site into the plasmid, and is thus expected to produce two fragments sized 3251 bp and 1144 bp when digested with EagI. pBR322, containing only one EagI restriction site, should linearize into a 4361 bp fragment after digestion. As shown in Figure 2, restriction enzyme digestion resulted in two visible bands for the pCAWK digest and one band for the pBR322 digest. The band size for the pBR322 digest was approximately 4361 bp, correlating with the expected results (Fig. 2). The sizes for the pCAWK digest however were approximately 4361 bp and 3300 bp, which does not match what was expected (Fig. 2). The 1144 bp fragment was not seen. Undigested pBR322 presented a lower band that could not be sized due to the lack of a supercoil ladder (Fig. 2). In contrast, the sequencing results showed that the insertion with the EagI restriction site was present in the *rop* gene (Fig. 3). Although the restriction mapping results did not correlate exactly with what was expected, taking the sequencing results into account as well suggests that pCAWK contains the correct insert.

Validation of the white/blue colony ratio screening assay to ensure that colony ratios correlate with plasmid copy ratios. As shown in figure 1, the white/blue colony ratio screening assay involves transforming the plasmids isolated from double transformants to measure the plasmid copy ratio. After plating the transformants on LB agar plates containing ampicillin, X-Gal and IPTG, each white colony will represent a pBR322 or pCAWK plasmid while each blue colony will represent a pUC19 plasmid. However, blue colonies can also occur after the co-transformation of pUC19 with pBR322 or pCAWK, resulting in an

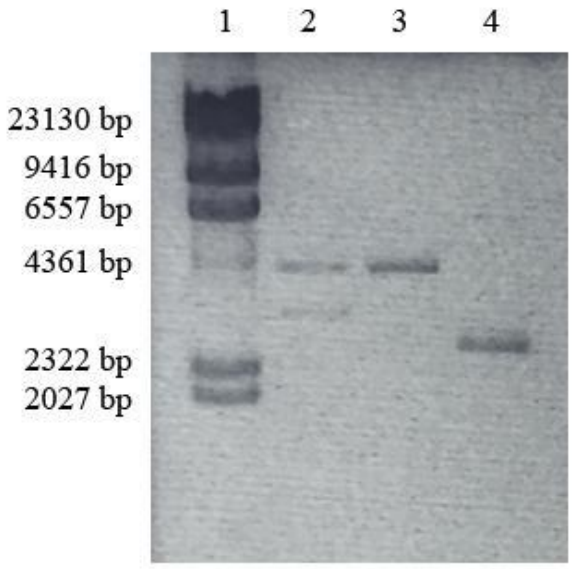

FIG 2 Restriction enzyme digestion of pCAWK and pBR322 with EagI followed by gel electrophoresis on 1.2% agarose gel. Lane 1: Lambda DNA/HindIII marker; lane 2: digested pCAWK; lane 3: digested pBR322; lane 4: undigested pBR322.

5′– CCGCAG CTGC**TAG**C**TAG**CTACGGCCGATCGATCGATCCAG CTGCCT –3′

3′– GGCGTC GAC**GAT**C**GAT**C**GAT**GCCGGCTAGCTAGCTAGGTC GACGGA –5′

FIG 3 Sequencing of the 34 bp insert in pCAWK using both forward and reverse primers. The underlined region represents the EagI restriction site and the multiple bolded regions represent stop codons. 6 bp flanking sequences are included.

underestimation of the number of pBR322/pCAWK-containing colonies. Therefore, the optimal plasmid amount at which double transformants are minimized must be determined. To do this, a 1:1 ratio of pBR322 and pUC19 were co-transformed at varying total plasmid amounts. These were then plated on LB agar plates containing tetracycline, X-Gal and IPTG to identify clones harboring pBR322 and to screen for cells that also took up pUC19. Successful transformation of pBR322 will allow for growth on tetracycline and successful co-transformation with pUC19 will result in a blue colony phenotype. After co-transformation with a total plasmid amount of 30 ng, large numbers of both white colonies containing pBR322 and blue colonies containing both pBR322 and pUC19 were obtained (Table 1). Decreasing the total amount of plasmid to 3 ng per transformation reduced the number of double transformants to only 2 colonies out of 71 (Table 1). Lower amounts of plasmid resulted in <30 colonies, an insufficient number of transformants for the assay (Table 1). Based on these results, 3 ng of plasmid seems to be the optimal plasmid amount of those tested at which double transformants is minimized.

After determining the optimal plasmid amount for the assay, an experiment was conducted to test whether or not our assay results correlate with the ratio of pBR322 and pUC19 used in transformation. pBR322 and pUC19 were combined at varying molar ratios and transformed using a total plasmid amount of 3 ng per transformation. These were then plated on LB agar plates containing ampicillin, X-Gal and IPTG; in this assay, clones harboring pBR322 are white and clones harboring pUC19 are blue. A graph was then generated comparing the observed ratios of white to blue colonies with the ratio of plasmid used for the transformation (Fig. 4). A positive slope was obtained, but the slope of the line is less that one, showing that plasmid ratio does not directly translate to colony ratio in the assay (Fig. 4). Therefore, when performing this assay with the experimental plasmids, the graph in Figure 4 should be treated as a standard curve that correlates the observed colony ratio with the actual plasmid ratio. A problem with using the graph as a standard curve is that because of the lack data points between the 7:3 and 9:1 plasmid ratios, the relationship between the two variables cannot be concluded as linear (Fig. 4). One possibility is that the relationship is linear with the highest data point being an outlier, but another possibility is that the relationship is exponential. This will not affect any qualitative conclusions that are made because a difference in colony ratios will still translate into a difference in plasmid ratios, but the quantitative magnitude of this difference will change depending on the relationship. The white/blue colony ratio screening assay requires a standard curve to correlate colony ratios with plasmid ratios but the standard curve generated in this study can only conclusively determine qualitative results.

Assessing the role of the *rop* gene on pBR322 exclusion when co-transformed with pUC19. Using the white/blue colony ratio screening assay, the plasmid copy ratio in pCAWK/pUC19 double transformants was compared against the ratio in pBR322/pUC19 double transformants. pBR322/pUC19 double transformants were obtained by co-transforming DH5α cells with pBR322 and pUC19, and plating on LB agar plates containing tetracycline, X-Gal and IPTG. Successful double transformants were identified by growth on tetracycline and blue phenotype. When this method was used to obtain pCAWK/pUC19 double transformants though, only white colonies were observed. Therefore, cells co-transformed with pCAWK and pUC19 were first plated on LB agar plates containing ampicillin, X-Gal and IPTG. In the absence of tetracycline, blue colonies could harbour both pCAWK and pUC19 or just pUC19. Testing the blue colonies for growth on tetracycline would identify double transformants. The plasmid copy ratios in the double transformants were then measured by isolating the plasmids and using 3 ng of plasmid per transformation for the white/blue colony ratio screening assay. Three separate pBR322/pUC19 double transformant colonies yielded only blue pUC19-containing colonies after the assay (Table 2). In comparison, the three pCAWK/pUC19 double transformant colonies gave rise to more white colonies than blue colonies at an average ratio of 2.78 after the assay. If the standard curve in figure 4 is assumed to be correct, a colony ratio of 2.78 correlates to a pCAWK:pUC19 ratio of 6.26. However, due to the problems with the standard curve, this plasmid ratio is probably incorrect. It is still the case though that the

pCAWK:pUC19 ratio is higher than the pBR322:pUC19 ratio, suggesting that the inactivation of the *rop* gene in pCAWK decreases the exclusion effect.

DISCUSSION

pBR322 has been observed to be selectively excluded from *E. coli* cells when co-transformed with pUC19, and the *rop* gene has been hypothesized to be one of the factors responsible for this phenomenon. This study aims to test the influence of the *rop* gene on relative plasmid exclusion and maintenance by using the *rop* gene-lacking pBR322-derivative pCAWK. After verification of the sequence of pCAWK, pBR322 and pCAWK were individually co-transformed with pUC19 into *E. coli* DH5α at equal ratios. The total plasmid was extracted from successful double transformants, and transformed again into competent *E. coli* DH5α cells at an amount that yields minimal double transformants, such that each cell takes up only one plasmid and exhibits the phenotype coded for by the plasmid. The white to blue ratio of the transformant colonies reflect the pBR322:pUC19 ratios and pCAWK:pUC19 ratios within the first set of *E. coli* transformants. Based on the assumption that the plasmid ratios result from plasmid maintenance and exclusion, and not from varying transformation efficiencies, we compared the two plasmid ratios to determine the influence of *rop* on plasmid exclusion.

First, we assessed the pCAWK sequence to confirm that it contains the correct insert within the *rop* gene. Based on the insert sequence, successful insertion into the gene should be adequate for inactivating it, since the insert length is 34 bp long and not a multiple of 3 (4). Additionally, the insert was designed to contain stop codons in both orientations (4). We verified pCAWK's sequencing by taking advantage of the EagI restriction cut site introduced by the insert in addition to the EagI cut site originally present in pBR322 (4). Although the gel electrophoresis result after pCAWK digestion did not correlate with what was expected, we concluded that pCAWK contains the correct insert when taking into account the restriction mapping results and sequencing results together (Fig. 2, Fig. 3). The higher band in the digested pCAWK lane had a size of approximately 4361 bp, which is similar to the 4395 bp linear form of pCAWK. This results from the digestion of only one of the two EagI restriction sites in pCAWK (Fig. 2). The presence of partially digested plasmid may have been due to the digestion time being too short. Other conditions, such as buffer and enzyme concentrations, may also have been suboptimal. The lower visible band in the digested pCAWK lane was approximately 3300 bp, corresponding to the expected size of the larger 3251 bp fragment resulting from digestion of both EagI sites on pCAWK (Fig. 2). Complete digestion of pCAWK should also yield a 1144 bp band, however, this was not visible on the gel (Fig. 2). Considering the predicted 3251 bp fragment was present, EagI most likely digested the plasmid at the correct locations, and the remaining 1144 bp of the plasmid may be present as well. The band may not have been visualized because at the same concentration, smaller fragments bind less ethidium bromide and thus have a lower band intensity. Sequencing results for pCAWK also confirmed that the plasmid contains the proper insert at the correct location in the plasmid (Fig. 3). The insert was incorporated in the opposite orientation than originally planned; the insert was supposed to contain stop codons in all reading frames for both orientations in case this occurred, but sequence analysis revealed that the stop codon for one of the reading frames in the reverse orientation did not exist (Fig. 3). However, the fact that the insert was present in the gene and the length of the insert is 34 bp and not a multiple of 3 is sufficient to confirm that *rop* is inactivated, since the *rop* gene is interrupted and the gene sequence downstream of the insert is shifted out of frame. pCAWK plasmid was considered verified and used in further experiments.

TABLE 1 Colony counts after co-transforming pBR322 and pUC19 into DH5α cells. Plasmid quantity was varied to determine the optimal amount at which transformation with both plasmids is minimized.

Total plasmid amount transformed (ng)	White colony count	Blue colony count	Total colony count
30	TNTC	176	TNTC
3	69	2	71
1	3	0	3
0.3	0	0	0
0.03	0	0	0

The standard curve for the white/blue colony screening assay yielded a positive relationship between plasmid ratio prior to transformation and plasmid ratio interpreted by colony phenotypes after transformation (Fig. 4). The R^2 value of 0.98 suggests that the linear model is a good fit for the data but an exponential relationship is also possible and intermediate data points between the 7:3 and 9:1 plasmid ratios should be included to complete the standard curve (Fig. 4). The value of the slope of the curve is less than one, indicating that the two ratios do not directly translate; the *E. coli* cells are more efficiently transformed with pUC19 than pBR322. One explanation is that pUC19 has a greater transformation efficiency than pBR322. This might be due to the fact that pUC19 is smaller than pBR322, and can thus be taken up by the cells easier. This hypothesis can be tested by performing separate transformations under the same conditions with pUC19 and pBR322, and comparing their transformation

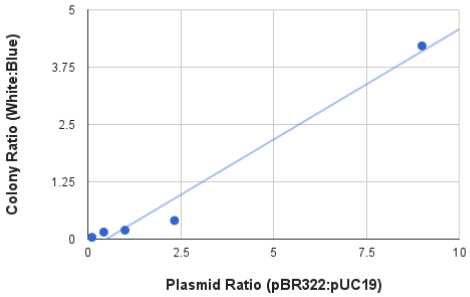

FIG 4 Standard curve for the white/blue colony ratio screening assay. Plasmid ratio is the pBR322:pUC19 molar ratio used for transformation (1:9, 3:7, 1:1, 7:3, 9:1). Colony ratio is the number of white to blue colonies that were presumed to contain pBR322 and pUC19 respectively.

efficiencies. It should be noted that some of the blue colonies presumed to only contain pUC19 may actually contain both pUC19 and pBR322, decreasing the counted number of pBR322-containing colonies and causing the colony ratio to be consistently lower than the plasmid ratio. Despite the effort to minimize double transformations by using only 3 ng of plasmid for transformation, two blue colonies containing both plasmids were observed from the colony count assay (Table 1). The standard curve was constructed under the assumption that no double transformations occurred, but even at 3 ng, 2.8% of the colonies contained both plasmids being tested (Table 1). In the standard curve assay, double transformants would have the same phenotype as single transformants containing pUC19. They would be counted as blue colonies and considered representative of a pUC19 single transformant, thus skewing the colony count ratios. Nevertheless, this small probability of double transformants can be taken into account when using the standard curve to interpret the original plasmid ratios in the experimental trial. Additionally, we expect the frequency of double transformation is similar between pBR322/pUC19 and pCAWK/pUC19 co-transformations since pBR322 and pCAWK only differ by 34 bp. In this case, double transformations would not be a confounding factor in any differences observed in the plasmid ratios between pBR322/pUC19 and pCAWK/pUC19 (4). One concern about the standard curve is the strong influence the 9:1 plasmid ratio data point has on the curve (Fig. 4). The data point was still included to provide a wider range of values to ensure that extrapolation is not necessary for the experimental values (Fig. 4). The reliability of the standard curve can be improved by increasing the amount of data points, especially in the large gap between the 7:3 and 9:1 values for the plasmid ratios (Fig. 4).

After co-transforming the cells with equal amounts of each plasmid, double transformants needed to be selected

TABLE 2 Colony counts after transforming DH5α cells with plasmid isolated from pBR322/pUC19 and pCAWK/pUC19 double transformants. Multiple samples were measured to determine the average plasmid ratio in the double transformants.

Sample	White	Blue	Ratio (White : Blue)	Plasmid ratio (pBR322 : pUC19 or pCAWK : pUC19)	Average plasmid ratio
pBR322 / pUC19 #1	0	73	0	0	
pBR322 / pUC19 #2	0	18	0	0	0 pBR322: 1 pUC19
pBR322 / pUC19 #3	0	31	0	0	
pCAWK / pUC19 #1	51	12	4.25	9.31	
pCAWK / pUC19 #2	78	41	1.90	4.44	6.26 pCAWK: 1 pUC19
pCAWK / pUC19 #3	122	56	2.18	5.02	

in order to assess the plasmid ratios within the cells following transformation. The original method of obtaining pCAWK/pUC19 double transformants was to select successful transformants on LB agar plates containing tetracycline, X-Gal and IPTG, but after multiple co-transformation attempts, only white colonies were obtained. This indicates that either pUC19 plasmids were not taken up by the cells, or that it was rapidly excluded from the cells following transformation. The likelihood of the cells failing to pick up pUC19 is low because this method was able to yield pBR322/pUC19 double transformants. The reason pUC19 was rapidly excluded following co-transformation with pCAWK may be because of the selective pressure of tetracycline. Both pUC19 and pCAWK confer ampicillin resistance, but only pCAWK mediates tetracycline resistance. Thus, pUC19 was likely unnecessary for cell growth and was lost during cell division. However, selection on tetracycline was necessary to ensure that pCAWK was also taken up by the cells. We therefore modified our selection method so that numerous blue colonies harboring pUC19 were first grown on LB agar plates containing ampicillin, X-Gal and IPTG. The clones were then screened on LB agar plates containing tetracycline to check for growth indicating the presence of pCAWK. This method allowed us to successfully isolate double-

transformants and proceed to isolate the total plasmid population from the cells.

Based on our co-transformation results with pUC19 and pBR322, the exclusion effect on pBR322 is very strong, as all of the colonies were blue, indicating that they all contain pUC19 (Table 2). Even considering the possibility of double transformants, most of the colonies likely only contain pUC19 as the frequency of double transformations was determined to be approximately only 2.8%. These results are expected because pUC19 is smaller than pBR322, contains the G to A mutation, and lacks the *rop* gene, all previously discussion plasmid factors that were proposed to promote plasmid maintenance (4). Compared to the pBR322/pUC19 double transformants, for which all colonies likely contained only pUC19, the pCAWK/pUC19 double transformants yielded many white colonies, resulting in an average pCAWK:pUC19 ratio of 6.26, which is considerably higher than the pBR322:pUC19 ratio (Table 2). This suggests that inactivating the *rop* gene mitigates the plasmid exclusion effect and agrees with the hypothesis that the *rop* gene contributes to plasmid exclusion. However, the observation that the pCAWK:pUC19 ratio exceeded 1 was not expected since pUC19 has the other proposed advantages of the G to A mutation and smaller plasmid size, in addition to the lack of *rop* which characterizes both plasmids. If any of the other proposed factors have an effect on plasmid maintenance, the ratio would be expected to be less than 1 because pCAWK would still be selectively excluded over pUC19. Even if the G to A mutation and plasmid size have no influence on the exclusion effect, the ratio would be expected to be approximately 1, signifying that neither plasmid is selectively excluded. There may be other factors present that produce a selective pressure to maintain pCAWK over pUC19 and may originate from the sequence inserted into the *rop* gene, although the insert was designed to not contain any meaningful sequences aside from the stop codons and the EagI restriction site (4).

Based on our restriction enzyme digest and sequencing results, pCAWK's *rop* gene was successfully inactivated by the insert and suitable for use to investigate the influence of the *rop* gene on plasmid exclusion. When validating the white/blue colony ratio screening assay, results indicated that 3 ng is the optimal total plasmid amount to use to minimize double transformations while yielding a countable range of colonies (30-300 cfu), and this value was used in the transformations for the experimental assays. Also, the standard curve yielded a positive linear curve appropriate for use to determine the original plasmid ratios based on the colony counts in the experimental trials. Observing only blue colonies for the pBR322/pUC19 double transformants indicate strict pBR322 exclusion and pUC19 maintenance, which aligns with previous expectations. The greater calculated plasmid ratio of 6.26 for the pCAWK/pUC19 double transformants suggests greater maintenance of pCAWK compared to pBR322 when co-transformed with pUC19. This supports the hypothesis of the *rop* gene contributing pB322 exclusion when co-transformed with pUC19.

FUTURE DIRECTIONS

Although this study gives support for the influence of the *rop* gene on the exclusion of pBR322 after co-transformation with pUC19, whether or not this factor is the sole factor resulting in pBR322 exclusion is unknown. Other possible factors include the G to A mutation in RNAII and plasmid size. To test the influence of the G to A mutation, site-directed mutagenesis can be used to create a pBR322-derived plasmid containing the mutation. In order to test the effect of plasmid size on pBR322 exclusion the plasmid can be reduced by either deleting the ampicillin or tetracycline resistance gene. This plasmid, as well as the plasmid with the G to A mutation, can then be tested using the screening assay described in this study to investigate the influence of the respective factors. An increase in plasmid ratio compared to pBR322:pUC19 will indicate that these factors also contribute to the exclusion effect, and the increases can be compared between experimental plasmids as well to rank the relative influence of each factor. To be able to quantitatively compare the factors though, the standard curve of the assay must be improved by including more data points.

The process of obtaining pCAWK/pUC19 double transformants was modified due to an inability of obtaining double transformants when plating on tetracycline. This was speculated to be because there was selective pressure to maintain pCAWK but not pUC19. However, if the ampicillin resistance gene of pCAWK is inactivated, the co-transformation reaction can be plated on media containing both tetracycline and ampicillin to obtain a selective pressure for both plasmids. This inactivation can also be introduced into the proposed G to A mutation plasmid if a similar problem of obtaining double transformants is encountered.

ACKNOWLEDGEMENTS

We would like to thank Dr. Oliver, Jia Wang and Celine Michiels for their guidance throughout the project. We also want to thank Airo *et al.* for constructing the pCAWK plasmid used. This project was financially supported by the Department of Microbiology and Immunology, University of British Columbia.

REFERENCES

1. **Lin-Chao S, Chen W, Wong T.** 1992. High copy number of the pUC plasmid results from a Rom/Rop-suppressible point mutation in RNA II. Mol Microbiol **6:**3385-3393.
2. **Atlung T, Christensen B, Hansen F.** 1999. Role of the Rom protein in copy number control of plasmid pBR322 at different growth rates in *Escherichia coli* K-12. Plasmid **41:**110-119.
3. **Smith M, Bidochka M.** 1998. Bacterial fitness and plasmid loss: the importance of culture conditions and plasmid size. Can J Microbio **44:**351-355.
4. **Airo A, Changizi F, Kim M, Wibowo J.** 2012. Construction of pCAWK, a novel pBR322-derived plasmid with insertional

inactivation of the *rop* gene. J Exp Microbiol Immunol **16:**129-135.

5. **Pamphile J, da Rocha C, Azevedo J.** 2004. Co-transformation of a tropical maize endophytic isolate of *Fusarium verticilloides* (synonym *F. monififorme*) with *gus*A and *nia* genes. Genet Mol Biol **27:**253-258.
6. **Sakai K, Sakaguchi J, Yamamoto M.** 1984. High-frequency cotransformation by copolymerization of plasmids in the fission yeast *Schizosaccharomyces pombe*. Mol Cell Biol **4:**651-656.
7. **Heine H.** 2003. Attempts at cloning pBR322Δ(1.1*rop*). J Exp Microbiol Immunol **4:**42-50.
8. **Sabaiduc S, Lo A.** 2008. Determination of exclusion effect in wild type and Rop deficient mutated pBR322 co-transformations. J Exp Microbiol Immunol **12:**118-122.
9. **Plaa N.** 2010. Determining the importance of a specific *rom* mutation in plasmid exclusion of pBR322 during pUC19 co-transformation. J Exp Microbiol Immunol **14:**112-115.
10. **Toh SY.** 2013. The study of exclusion effect of pBR322 using its *rop*-inactivated mutant, during co-transformation with pBR322 and pUC19: plasmid copy number does not relate to the exclusion of pBR322. J Exp Microbiol Immunol **17:**109-114.
11. **Hancock REW.** 19 September 1999. Hancock laboratory methods. Department of Microbiology and Immunology, University of British Columbia, British Columbia, Canada. http://www.cmdr.ubc.ca/bobh/methods.htm.