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Copy Number of ColE1-type Plasmids Decreases When *rop* is Supplied in *trans* on a Separate Plasmid

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Previous studies have shown that in *Escherichia coli* co-transformed with two different plasmids, the expression of the Rop protein from one plasmid can act in *trans* to decrease the copy number of the other plasmid, an effect referred to as plasmid exclusion. These studies relied on previous findings on ColE1-type plasmids showing that copy number is primarily regulated by the RNA II transcript, which serves as a primer for plasmid replication, the RNA I transcript, which binds and sequesters RNA II, and the Rop protein, which stabilizes the RNA I : RNA II hybrid. However, these studies, which used the pUC19, pBR322, and pCAWK vectors, did not account for the influence of an RNA II point mutation that was present in pUC19, but absent in both pBR322 and pCAWK. Hence, we decided to use the pAPA3 plasmid in lieu of pUC19, to control for the effect of the RNA II point mutation. We hypothesized that by controlling for the RNA II point mutation with the use of pAPA3 plasmid, the presence of Rop would result in greater levels of exclusion of the lower copy number plasmids in co-transformed cells. To test this hypothesis we generated co-transformants in *Escherichia coli* DH5α containing either pBR322 or pCAWK and pAPA3 plasmids. Plasmids from co-transformants were isolated and copy numbers were estimated by transforming *E. coli* DH5α and selecting for growth using antibiotic selection. The ratio of the number of transformants on the selective media was used as a proxy for the ratio of pAPA3 to pBR322 or pCAWK in the co-transformed cells. We observed that the pAPA3:pCAWK ratio was 6 times greater than the pAPA3:pBR322 ratio, suggesting that the expression of Rop reduces plasmid exclusion. Based on this observation, we propose that the Rop expressed by pBR322 acted in *trans* to reduce pAPA3 copy numbers, effectively increasing the ratio of pBR322 relative to pAPA3 in the cell. Conversely, the absence of the *rop* gene on both pAPA3 and pCAWK allowed pAPA3 to replicate to a higher copy number, thereby reducing the ratio of pCAWK relative to pAPA3.

Plasmids are independently replicating genetic elements that can play an important role in determining host fitness in different environments (1). Plasmid vectors have also been used in the laboratory setting to manipulate genes of interest, and have been essential to the study of genetic systems in microbiology (2). Commonly used vectors in *Escherichia coli* include pBR322 and pUC19 from the ColE1 family of plasmids (2). This family of plasmids are generally characterized by the presence of an RNA II gene, which codes for the primer used in plasmid replication (3). Plasmid replication needs to occur at least once per generation in order for a given plasmid to be maintained within a population of actively dividing cells (2). However, excessive plasmid production will physiologically strain the host cell due to the metabolic burden imposed by plasmid replication (2, 4, 5). Hence, copy number is subject to tight regulation by elements found within the plasmids themselves (2). The copy number of ColE1-type plasmids is known to be regulated by two elements: the RNA I transcript and the *rop* gene (1, 4). Both act on the RNA II transcript, which in its active form serves as the primer for plasmid replication by binding at the *ori* or origin of replication (6, 7). RNA I interacts with RNA II to form an RNA:RNA hybrid that sequesters RNA II and prevents its conversion into the active form (2, 4, 6, 7). Rop binds to this RNA I:RNA II complex, stabilizing the interaction between them and further inhibiting plasmid replication (2, 4, 6). It has also been observed that plasmid size negatively correlates with copy number (2, 4, 6, 7).

Previous studies focusing on understanding copy number regulation of pBR322 and pUC19 co-transformed into *E. coli* found that after several rounds of cell division, pUC19 was maintained in the host population while pBR322 was lost in a phenomenon called "plasmid exclusion" (8, 9). This is in contrast to cells singly transformed with either pUC19 or pBR322, where the plasmids were maintained in the population at a high copy number even after several generations (10, 11).

Mo *et al*. attempted to study the effect of Rop expression on plasmid exclusion by using cells co-transformed with pUC19 and pBR322 or pCAWK (8). Notably, pUC19 lacks a functional *rop* gene and contains a point mutation in its RNA II sequence, while pCAWK is a derivative of pBR322 with a *rop* gene that has been inactivated by a 34 bp insert (10, 12). Their experimental approach involved co-transformed cells being selected with a blue/white colony screening assay. pUC19, which contains the *lacZα* gene, produced blue colonies when grown on LB containing X-gal and IPTG, while pBR322 and pCAWK produced white colonies. Hence, the assay allowed them to determine the ratio of pBR322 or pCAWK to pUC19 in the co-transformed cells. While their results indicate that Rop expression contributed to the exclusion of the lower copy number plasmid, their approach did not evaluate the role of the pUC19 RNA II point mutation, which has been observed to have an effect on plasmid copy number (8, 10).

In this study, we aimed to more clearly study the effects of *rop* on plasmid exclusion with the use of the pAPA3 plasmid, a pUC19 derivative that does not possess the point mutation in its RNA II sequence (13). The use of the pAPA3 plasmid would ensure that all co-transformed plasmids have similar RNA II sequences, and allow for study of the effect

of Rop alone (10). We hypothesized that, similar to the observations of Mo *et al.*, the inactivated *rop* gene on pCAWK allows it to be expressed in higher copy numbers than pBR322 when co-transformed with pAPA3 (8). We also predicted that the absence of the RNA II point mutation (which reduces plasmid replication inhibition) on pAPA3 would reduce its copy number relative to pBR322 and pCAWK, in comparison to the ratios of pBR322 and pCAWK to pUC19 as observed by Mo *et al.*, where pBR322 was completely excluded and the pCAWK:pUC19 ratio was 6.26:1 (8).

MATERIALS AND METHODS

Strains and plasmids. An *E. coli* HB101 strain containing the pBR322 plasmid and an *E. coli* DH5α strain containing the pCAWK plasmid were obtained from the University of British Columbia (UBC) Department of Microbiology and Immunology strain collection. An *E. coli* DH5α strain containing the pAPA3 plasmid was a gift from Tanya Parish and was obtained from the Addgene plasmid repository (Addgene plasmid # 24777) (13).

Plasmid isolation. *E. coli* strains containing pAPA3, pBR322, and pCAWK were incubated at 37ºC and shaken at 200 rpm for 16 to 20 hours in 5 mL of Luria Bertani (LB) broth containing 100 µg/mL ampicillin. Plasmids were isolated using the Beatty Lab protocol. Plasmid concentrations was measured using the NanoDrop 2000c spectrophotometer.

Restriction enzyme digestion and agarose gel electrophoresis. pBR322 and pCAWK were digested with the EcoRI and NdeI restriction enzymes, while pAPA3 was digested with NdeI only. All restriction enzymes used in this study were purchased from New England Biolabs (NEB), and all digests were carried out for 1 hour in Cutsmart Buffer 3.1. Digests were run with undigested plasmids and a 1 kb linear DNA ladder from NEB on a 1% agarose gel in TAE buffer at 170 V for 30 minutes. Fragments were visualised by staining with ethidium bromide (EtBr) for 20 minutes.

Comparison of genetic elements among different plasmids. Complete plasmid DNA sequences for pAPA3 and pBR322, as well as the sequences of relevant genetic elements on each plasmid, were obtained from AddGene. The complete pCAWK sequence was generated by adding the 34 bp insert created by Airo *et al.* into the pBR322 *rop* gene sequence (12). The Benchling plasmid annotation tool was used to map the relevant genetic elements onto each plasmid sequence (see supplementary Fig. S1). Bl2seq utilizing the blastn algorithm was used to align and compare the entire DNA sequences of pBR322 and pCAWK, as well as the DNA sequences of the *ori* and the *ampR*, RNA I, and RNA II genes and promoters of pAPA3 and pBR322/pCAWK. It was also used to align and compare the pBR322 *rop* gene DNA sequence to that of the entire pAPA3 plasmid. Bl2seq utilizing the blastp algorithm was then used to align and compare the AmpR sequences of pAPA3 and pBR322/pCAWK, which were derived from their respective gene sequences using the Benchling auto-translate tool.

Preparation of competent cells. A modified version of the Inoue Ultra-Competent Cell Protocol was used to prepare competent *E. coli* DH5α cells (14). Cells were grown in 25 mL super optimal broth (SOB) at 37ºC while shaken at 250 rpm for 6 hours to prepare seed cultures. A 4 mL aliquot of the seed culture was transferred into 250 mL SOB and incubated overnight at 37ºC and shaken at 200 rpm to an OD_{600} of 0.5 - 0.6. The cells were subsequently placed on ice for 10 minutes, then centrifuged at 2,500 rcf for 10 minutes at 4ºC. After discarding the supernatant, cells were resuspended in 80 mL of chilled Inoue transformation buffer (ITB) and centrifuged again under the same conditions. The supernatant was removed, and cells were resuspended in 20 mL chilled ITB. After adding 1.5 mL dimethyl sulfoxide (DMSO), cells were divided into 1 mL aliquots and flash frozen by immersion in dry ice and EtOH. Aliquots were stored at -80ºC.

Transformation of competent cells. Transformation was carried out by following a modified version of the Inoue Ultra-Competent Cell Protocol (14). Competent cells were thawed, divided into 50 µL aliquots, and stored on ice for 10 minutes. Plasmids were added to the competent cells, which were then mixed and left on ice for 30 minutes. Cells were heat shocked in a 42ºC water bath for exactly 90 seconds, then rapidly transferred to an ice bath for 2 minutes. After adding 800 µL of pre-warmed super optimal broth with catabolite repression (SOC) medium, subsequently cells were placed in a 37ºC water bath for 10 minutes to be brought to incubation temperature. Cells were then incubated at 37ºC while shaken at 150 rpm for 45 minutes. LB-agar containing the desired antibiotic(s) were then spread plated with 100 µL aliquots of transformed cells, and incubated overnight at 37ºC.

Minimum inhibitory concentration assay. A minimum inhibitory concentration (MIC) assay was conducted for pAPA3, pBR322, and pCAWK using a modified version of the Hancock agar and broth dilution method (15). Four isolated colonies from single transformants, spread plated on LB-agar containing 100 µg/mL ampicillin, were used to inoculate 5 mL LB broth containing 100 µg/mL ampicillin. Cultures were grown overnight at 37ºC while shaken at 150 rpm. A polyethylene 96-well microtiter plate containing 50 µL of LB with varying concentrations of ampicillin, gentamicin, and tetracycline were inoculated with 50 µL aliquots of each single transformant to determine the MIC of each antibiotic for each plasmid. The final concentrations of gentamicin and tetracycline ranged from 0 - 50 µg/mL, while those of ampicillin ranged from 0 - 2000 µg/mL. Additionally, wells containing 100 µL of LB broth served as negative controls, while wells containing 50 µL LB broth and inoculated with 50 µL aliquots of transformed cells served as positive controls.

Colony counts of single transformants. Single transformants were generated with pAPA3, pBR322, and pCAWK plasmids by transforming aliquots of competent *E. coli* DH5α with 100 ng of plasmid DNA. All transformants were spread plated on LB-agar containing 100 µg/mL ampicillin. Colony counts obtained were used as a proxy to compare the relative transformation efficiencies for each plasmid.

Preparation of double transformants using sequential transformation. pAPA3/pBR322 and pAPA3/pCAWK double transformants were prepared by initially transforming 100 ng of pBR322 or pCAWK into competent *E. coli* DH5α. Transformants were plated on LB-agar containing 100 µg/mL ampicillin, and isolated colonies were picked and prepared into competent cells, substituting SOB with SOB containing 100 µg/mL of ampicillin to ensure plasmid maintenance. Each strain was subsequently transformed with 100 ng of pAPA3 and plated on LB-agar containing 20 µg/mL of gentamicin and tetracycline.

Colony ratio screening assay for the characterization of co-transformants. Plasmids were isolated from 5 mL cultures of LB broth containing 100 µg/mL of ampicillin, inoculated with double transformants and grown overnight at 37ºC while shaken at 150 rpm. Aliquots of competent *E. coli* DH5α were transformed with 200 ng and 400 ng of plasmid DNA from each isolate. Transformants were allowed to recover for 90 instead of 45 minutes. Cells were then spread plated in 100 µL aliquots onto LB-agar containing either 10 µg/mL tetracycline, 20 µg/mL gentamicin, or both 20 µg/mL gentamicin and 10 µg/mL tetracycline, and colony counts were performed after overnight incubation at 37ºC. Standard curves for the transformation efficiency of pAPA3, pBR322, and pCAWK were generated by

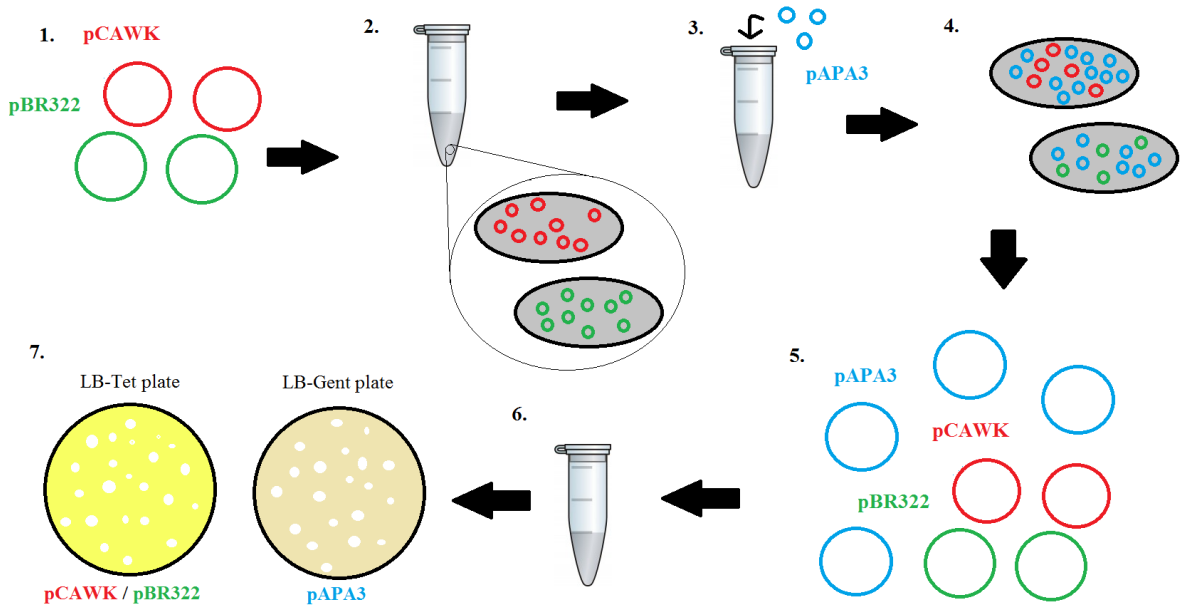

FIG. 1 Work flow for sequential transformation and colony ratio screening assay. 1. Isolated pBR322 (green) and pCAWK (red) from single transformants. 2. Generated single transformants containing pBR322 or pCAWK. 3. Converted single transformants into competent cells and sequentially transformed with isolated pAPA3. 4. Selected for pAPA3/pBR322 and pAPA3/pCAWK co-transformants by spread plating on LB-Gent+Tet. 5. Isolated plasmids from co-transformants. 6. Transform individual plasmids into competent cells. 7. Select for singly transformed cells on LB-Gent and LB-Tet.

transforming competent *E. coli* DH5α with 0, 30, 60, 100, and 200 ng of plasmid DNA. The general workflow of the sequential transformation and screening assay is summarized in Figure. 1.

RESULTS

Verification of pAPA3, pBR322, and pCAWK via restriction enzyme digests. Restriction digests were conducted on pAPA3 (5.9 kb), pBR322 (4.4 kb) and pCAWK (4.4 kb), and the resulting fragments were run on a 1% agarose gel in order to verify the plasmid identities based on expected cut sites and band sizes. Digestion of pAPA3 with NdeI resulted in 2 bands that were approximately 2.2 kb and 3.7 kb in size (Fig. 2). Digestion of pBR322 and pCAWK with NdeI and EcoRI resulted in 2 bands that were approximately 2.1 kb and 2.3 kb in size, although the quality of the printed gel photo makes it difficult to resolve these bands (Fig. 2). Hence, the results illustrated in Figure 2 indicate that the number and size of the digested bands were largely consistent with the expected values and confirm the identities of pAPA3, pBR322, and pCAWK. Surprisingly, the bands of the undigested plasmids closely corresponded to their actual sizes, which would be more consistent with linearized plasmids than supercoiled, closed circular, or open-nicked circular plasmids (Fig. 2). However, the absence of obvious smears on the gel indicates that this was not due to DNase contamination. Furthermore, the qualitative analysis of the relative intensities of the undigested plasmid bands illustrated in Figure 2, lanes 2 - 4, suggest that the plasmid isolation procedure yielded substantially more pAPA3 than pBR322 or pCAWK. These relative intensities were also consistent with our spectrophotometric analysis, which showed the concentration of pAPA3 to be 4 - 5 fold higher than that of pBR322 and pCAWK (see supplementary Table S1). While Figure 2, lanes 3 and 4 indicate that there was a higher yield of pCAWK compared to pBR322, lanes 6 and 7 indicate that roughly equal amounts of each plasmid were isolated. This inconsistency was resolved by consulting spectrophotometric data, which showed that roughly equal amounts of pBR322 and pCAWK were

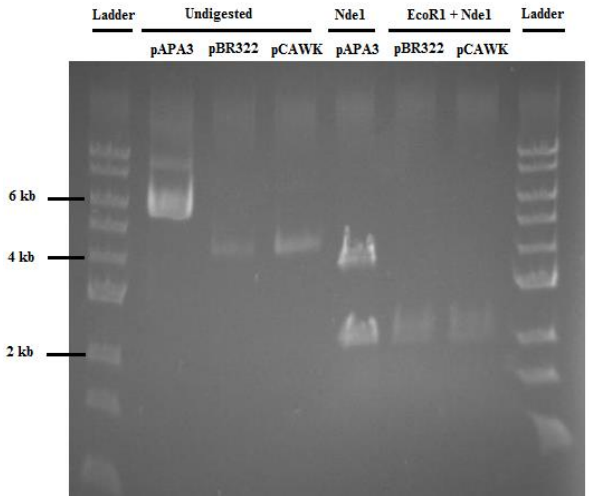

FIG. 2 Agarose gel results to validate pAPA3, pBR322 and pCAWK plasmid identities. Samples and ladders were run on a 1.0% agarose gel at 170 V for 30 minutes in 1 x TAE buffer.

isolated (see supplementary Table S1). Hence, the differing intensities in Figure 2, lanes 3 and 4 may have been due to human error in loading the sample, especially considering the small volumes involved.

Sequence comparison of pAPA3, pBR322, and pCAWK. Table 1 summarizes the bl2seq multiple sequence alignment results comparing the DNA sequences of relevant genetic elements on the 3 plasmids. Since a blastn comparison of the entire sequence of pBR322 and pCAWK revealed a 99% sequence identity, with the differences due to the 34 bp insert disrupting the *rop* gene of pCAWK (not shown on table), the results in Table 1 essentially represent sequence comparisons between pAPA3 and pBR322/pCAWK. Consistent with the expected values the sequences of the *ori* and the RNA I and RNA II genes of the 3 plasmids were identical to one another, with no gaps and 100% sequence identities (Table 1). On the other hand, the amp^R gene had 99% sequence identity representing a 2 bp difference between pAPA3 and pBR322/pCAWK (Table 1). A comparison of their respective protein sequences via blastp likewise revealed a 99% sequence identity (not shown on table). More specifically, the changes in the amino acid sequence of pAPA3 were $Ile^{82} \rightarrow Val^{82}$ and $Val^{182} \rightarrow Ala^{182}$. Finally, a bl2seq multiple sequence alignment between the pBR322 *rop* DNA sequence and the entire pAPA3 plasmid DNA sequence revealed that the only region that had a high degree of sequence similarity was 36 bp long. Since the functional *rop* gene of pBR322 is 192 bp long, it was stipulated that pAPA3 does not have a functional *rop* gene.

Characterization of antibiotic resistance conferred by pAPA3, pBR322, and pCAWK via the MIC assay. The MIC assay was conducted in order to determine the effective concentration of antibiotics to select for cells containing specific plasmids. Transformants were considered resistant to a particular concentration of antibiotics if wells became turbid after 20 hours of incubation, and were considered sensitive if wells remained clear. Qualitative observations of the microtiter plate showed that cells transformed solely with pAPA3 are resistant to the 100 µg/mL of ampicillin and 20 µg/mL of gentamicin, but sensitive to the 10 and 20 µg/mL of tetracycline used in our experiments. On the other hand, cells transformed solely with pBR322 or pCAWK are resistant to the 100 µg/mL of ampicillin and 10 and 20 µg/mL of tetracycline, but sensitive to the 20 µg/mL of gentamicin used in our experiments. In general, results were consistent with expectations, as pAPA3 contains ampicillin and gentamicin resistance genes while pBR322 and pCAWK both contain ampicillin and tetracycline resistance genes. All negative control wells remained clear while all positive control wells were observed to be turbid after incubation.

TABLE 1 Bl2seq multiple sequence alignments of pertinent genetic elements on pAPA3 and pBR322/pCAWK. Note that cds stands for coding sequence

Element	Gaps (bp)	Identity
pMB ori sequence	0	100%
RNA I cds	0	100%
RNA I promoter	0	100%
RNA II cds	0	100%
RNA II promoter	0	100%
AmpR cds	0	99%
AmpR promoter	0	100%

Colony counts of single transformants to compare transformation efficiencies. Colony counts were performed on single transformants in order to compare the transformation efficiencies of pAPA3, pBR322, and pCAWK into *E. coli* strain DH5α. The transformation efficiency of pAPA3 was at least 2 fold higher than the transformation efficiencies observed for cells transformed with pBR322 or pCAWK (Fig. 3a). Figure 3b reinforces the findings of Figure 3a by illustrating that transformations with pAPA3 consistently produced a high number of CFUs, even when the plasmid stocks used were isolated from independent experiments and shows the high numbers of transformants obtained. Figure 3c shows that the number of CFUs produced by cells transformed with pCAWK were higher than those transformed with pBR322, although substantial variations in CFUs were present among transformations. Figure 3c further indicates that constructing standard curves for transformation efficiency were largely inconsistent between each day, deeming it necessary for the creation of standard curves to be performed in parallel to the assays used in plasmid copy determination, to control for this day to day variation in CFUs. Taken together, these results suggest that the transformation efficiency of pAPA3 is at least 2 fold higher than that of pBR322 and pCAWK (Fig. 3a, 3b). Our data also suggests that the transformation efficiency of pCAWK is 50% higher than that of pBR322 (Fig. 3a, 3c).

Development of standard curves for the colony ratio screening assay. The standard curves illustrated in Figure 4a-c were produced to relate the number of CFUs with the amount of plasmid DNA added in each transformation. By transforming DNA of various concentrations into competent cells, we were able to construct curves based on colony counts that served as a proxy for transformation efficiency. The number of colonies obtained from the colony ratio screening assay were used to determine the DNA concentration of each plasmid using these standard curves. It should be noted that colony counts obtained from transforming 100 ng of pCAWK DNA were removed from the data set, as these were deemed to be inconsistent with the general trend. Figure 4d illustrates the relative differences in plasmid transformation efficiencies by superimposing the best fit lines of the 3 standard curves. All standard curves showed a linear relationship between the amount of DNA added and the number of CFUs. The transformation efficiencies of pAPA3 and pCAWK were both about 9.5 cfu/ng of DNA (Fig. 4d). The transformation efficiency of pBR322 was approximately 4.3 cfu/ng (Fig. 4d).

The colony ratio screening assay to determine that Rop decreases plasmid copy number when supplied *in trans*. Having determined the transformation efficiency of each plasmid, we proceeded to measure the ratio of pAPA3 to either pBR322 or pCAWK when co-transformed into competent *E. coli* DH5α cells. Co-

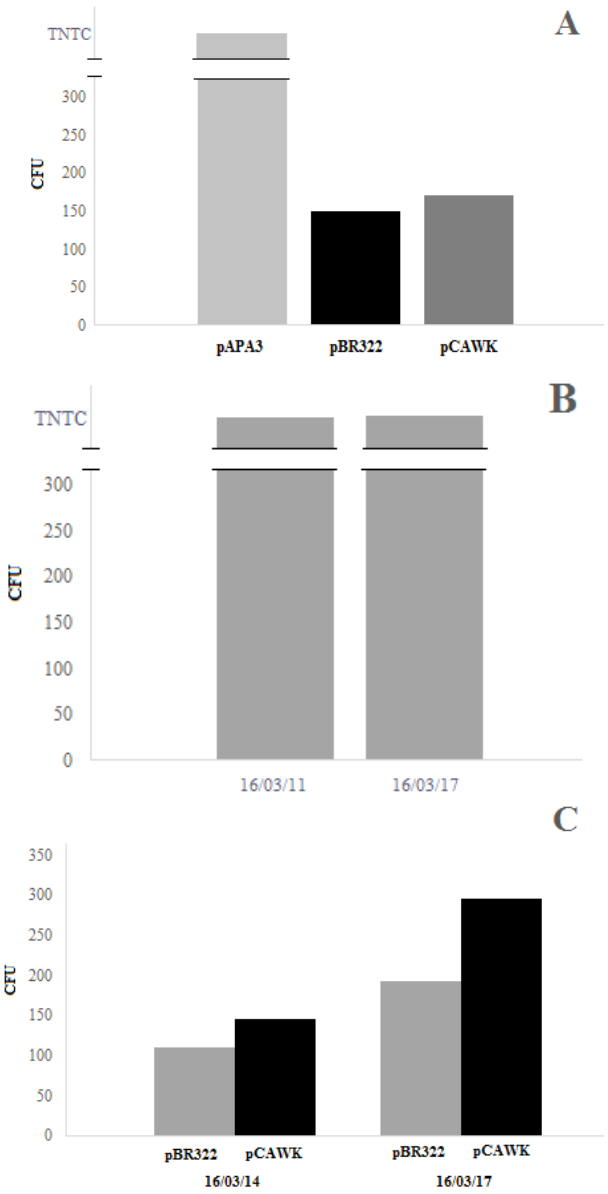

FIG. 3 Colony counts for pAPA3, pBR322, and pCAWK used to estimate and compare transformation efficiencies among the 3 plasmids. (A) Colony counts of single transformants of pAPA3, pBR322, and pCAWK representative of multiple replicates, using stocks of plasmids prepared on different dates. **(B)** Colony counts of transformations with pAPA3 done on different days using different stocks of the plasmid. **(C)** Colony counts of transformations with pBR322 and pCAWK done on different days using different stocks of each plasmid. For all treatments, 100 ng of plasmid DNA was transformed into competent cells using the Inoue protocol.

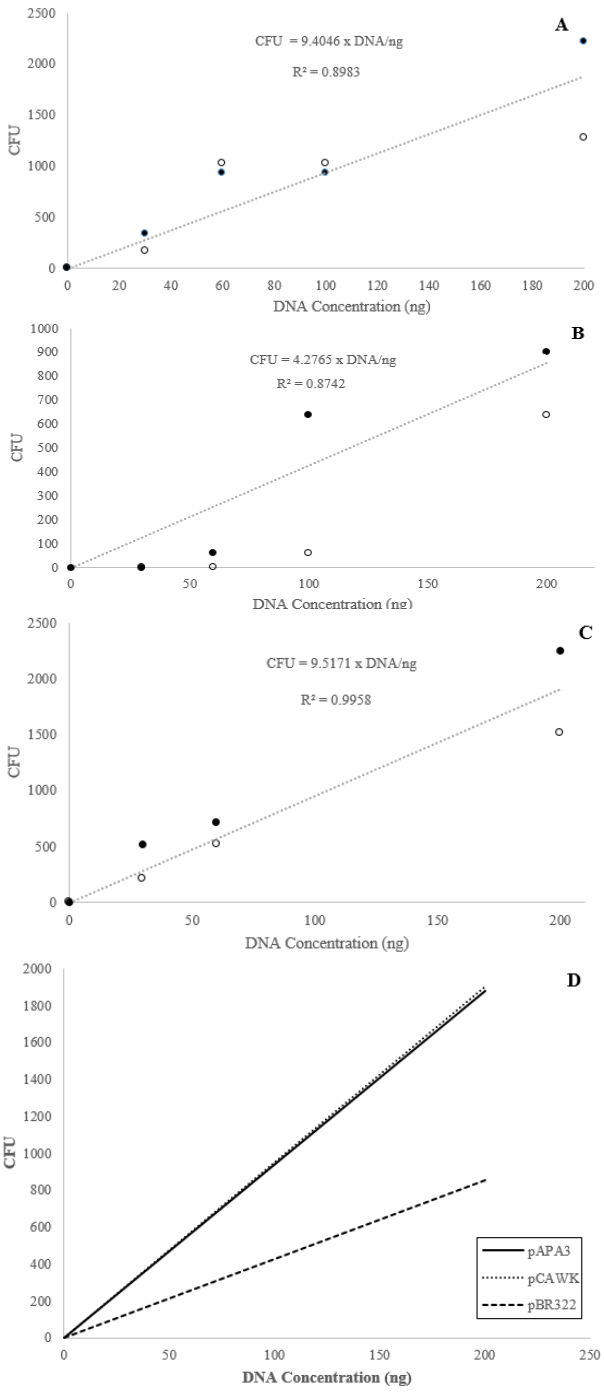

FIG. 4 Standard curves of the transformation efficiencies of (A) pAPA3, (B) pBR322, and (C) pCAWK. (D) Superimposition of the standard curves of each plasmid as a comparison of their transformation efficiencies. Note that colony counts for (C) observed at the 100 ng concentration were removed in the creation of the best fit line. Open and closed circles on (A), (B), and (C) represent data points derived from 2 replicates.

transformed cells were grown overnight with the common antibiotic, ampicillin, and the total plasmid content of each culture was isolated. The isolated plasmids were diluted and re-transformed into competent *E. coli* DH5α cells. As a control for possible transformants carrying both plasmids, we spread a 100 µL aliquot of the transformed cells onto plates containing both gentamicin and tetracycline. Half of the remaining transformed cells were spread as 100 µL aliquots onto LB-Gentamicin plates, while the other half were spread as 100 µL aliquots onto LB-Tetracycline plates. Each single antibiotic containing plate served to select for the relevant plasmid(s) and

generate CFUs for colony counts. CFUs generated on by each plasmid were counted and divided by their respective transformation efficiency generated by the standard curves to obtain the relative concentration of each plasmid present in the co-transformed cells. As shown in Table 2, it was observed that higher amounts of pAPA3 were present in cells co-transformed with pCAWK and pAPA3 compared to those co-transformed with pBR322 and pAPA3. More specifically, regarding the 200 ng data points, it was found that the amount of pAPA3 within co-transformed cells was found to be approximately 5.9 and 36 times greater than pBR322 and pCAWK, respectively. This suggests that there is greater exclusion of pCAWK compared to pBR322 in co-transformed cells.

TABLE 2 Estimated plasmid concentration ratios measured for pAPA3 + pCAWK and pAPA3 + pBR322 sequentially transformed cells.

	pAPA3 + pBR322 Co transformants			pAPA3 + pCAWK Co transformants		
Total Homogenous Plasmid Transformed (ng)	Estimated pAPA3 DNA (ng)	Estimated pBR322 DNA (ng)	pAPA3/ pBR322 ratio	Estimated pAPA3 DNA (ng)	Estimated pCAWK DNA (ng)	pAPA3/ pCAWK ratio
200	85.6 ± 5.7	14.6 ± 2.4	5.9 ± 2.4	10.8 ± 2.3	0.3 ± 0.3	36.0 ± 7.7
400	8.7 ± 0.8	2.0 ± 1.1	4.4 ± 0.7	16.3 ± 0.5	1.5 ± 0.3	10.9 ± 1.7

DISCUSSION

Building on the results of Mo *et al.*, this study sought a more detailed examination of the effect of *rop* in the exclusion of co-transformed plasmids. To this end, we decided to use pAPA3 instead of pUC19 for our experiments, since it also lacks the *rop* gene yet does not possess the RNAII point mutation present in pUC19 (10). By sequentially transforming cells with pAPA3 into pBR322 or pCAWK containing cells, we compared the influence of *rop* on individual plasmid copy number.

As pCAWK lacks a functional *rop* gene, we expected that it would have a higher copy number when co-transformed with pAPA3 compared to pBR322, since *rop* would have a dampening effect on plasmid copy number. This would be similar to what was observed by Mo *et al*., where pCAWK was observed to have a higher copy number than pBR322 when co-transformed with pUC19 (8). However, the colony ratio screening assay results were contradictory to our hypothesis, since the pAPA3:pCAWK ratio is at least 2.5 fold higher than the pAPA3:pBR322 ratio (Table 2). It should be noted that we observed a total of 27.8 ng of plasmid DNA was transformed for cells transformed with 400 ng plasmid DNA, compared with 111.3 ng for cells transformed with 200 ng (Table 2). This might suggest that the addition of 400 ng of plasmid DNA represents a saturating amount which resulted in lower transformation efficiencies, as observed in the literature when cells are transformed with high concentrations of DNA (16). Therefore, the 200 ng concentrations of total plasmid transformed were deemed to be a better measure for comparing the pBR322 and pCAWK ratios with pAPA3, where pAPA3:pCAWK was observed to be 6 times higher than pAPA3:pBR322 (Table 2).

These findings led us to develop a model to explain the role of Rop expression during co-transformation (Fig. 5). We propose that Rop is exerting its effect by inhibiting plasmid replication *in trans* and thereby reducing pAPA3 plasmid copy numbers in pAPA3 and pBR322 containing cells. Rop is known to be able to repress plasmid replication in *trans* with incompatible plasmids, and therefore is hypothesized to stabilize the RNA II:RNA I complex, inhibiting replication of both pBR322 and pAPA3 (6). We speculate that this prevents pAPA3 from reaching the copy number it would normally reach when transformed into cells. On the other hand, in cells co-transformed with pCAWK and pAPA3, cells were devoid of Rop which allowed pAPA3 to reach higher copy numbers than in cells co-transformed with pBR322, therefore resulting in relatively higher ratios observed for pBR322 than pCAWK. In summary, the absence of Rop increased copy numbers of pAPA3 residing in cells with pCAWK compared to cells also containing pBR322.

We speculate that the high ratio of pCAWK to pUC19 observed in Mo *et al.*'s study was due to the lack of Rop inhibition on pCAWK replication. While pBR322 plasmid replication was presumably inhibited by Rop while co-existing with pUC19 which was present in such high copy numbers, pBR322 was excluded from cells. Furthermore, as past studies suggest that Rop might only allow for suboptimal interactions with the RNA I:RNA II hybrid transcribed by pUC19, Rop in pBR322 and pUC19 containing cells might have only partially inhibited pUC19 plasmid replication initiation, still allowing it to reach relatively high copy numbers and exclude pBR322 in cells (10).

In order to best account for the different transformation efficiencies for the plasmids, standard curves for plasmid copy number determination were performed in parallel. The plasmids were singly transformed in parallel with the isolated DNA from co-transformed cells and plated in duplicate. As seen in Figure 4a-c, the degree of variation in colony counts between duplicates are not relatively large, and therefore we assume that this method is effective in controlling for the large variations in transformation efficiency observed between plasmids when transformation were performed on separate days detailed in Figure 3c.

Finally, it should also be noted that pAPA3 consistently showed higher transformation efficiency compared to pBR322 and pCAWK, even when selecting on plates containing the same antibiotic. This result suggests that the high transformation efficiency of pAPA3 is due to a characteristic of the plasmid itself and not due to experimental variables. We speculate that these higher transformation efficiencies are due to pAPA3 having a higher copy number than pBR322 and

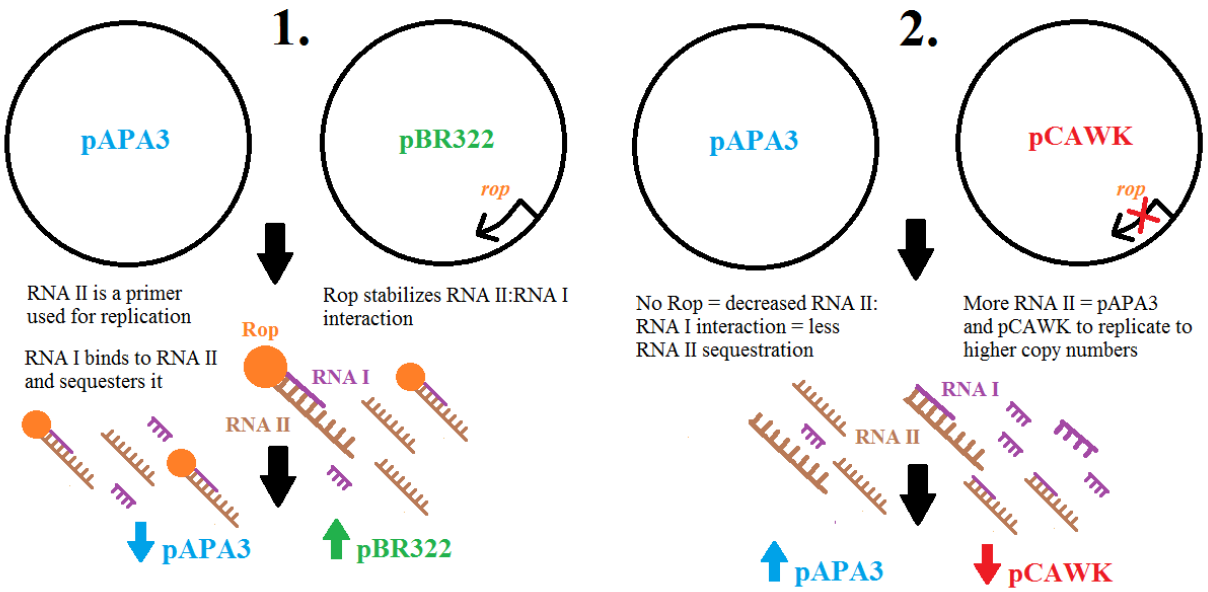

FIG. 5 Model of how Rop expression affects plasmid copy number in co-transformed cells. (1) pBR322 expresses Rop, which can act in *trans* and stabilizes the RNA II:RNA I complex from either plasmid, effectively lowering the RNA II available for pAPA3 and pBR322 replication. Since pAPA3 normally does not express Rop while pBR322 does, Rop expression effectively lowers the copy number of pAPA3 relative to pBR322. (2) pAPA3 and pCAWK both lack a functional *rop* gene, thus stabilization of the RNA II:RNA I complex is absent, allowing for a greater overall pool of RNA II for replication. Without Rop expression, replication of pAPA3, which is a high copy number plasmid, is less inhibited and hence able to exclude pCAWK to a greater degree than pBR322, both of which are medium copy number plasmids.

pCAWK. During transformation, pAPA3 may be rapidly replicated during the recovery phase following heat shock, thereby reducing the likelihood that the plasmid is lost during cell division and increase the likelihood of generating antibiotic resistant cells. The differences between the (amp^R) genes of pAPA3 and pBR322/pCAWK, which translated to a 2 amino acid difference in their respective β-lactamase proteins, may have also affected the ampicillin resistance conferred by each plasmid, although we were unable to confirm this (Table 1). Taken together, we speculate that the high transformation efficiency of pAPA3 may be due to its high copy number and/or the mutations in its amp^R gene.

In conclusion, pAPA3 was shown to be suppressed in *trans* by Rop when co-transformed with pBR322, and this inhibition allowed for relatively higher amounts of pBR322 compared with pCAWK when using pAPA3 as a baseline. This was evident by the 6-fold increase in the ratio of pAPA3 to pCAWK compared to the ratio of pAPA3 to pBR322 (Table 2). Hence, the expression of Rop allowed for a lower degree of exclusion in cells co-transformed with ColE1-type plasmids.

FUTURE DIRECTIONS

While the current study controlled for the potential effects of the pUC19 RNA II point mutation on plasmid exclusion, it did not account for the influence of plasmid size. This might be significant as pAPA3 is approximately 1.6 kb larger than pBR322 and pCAWK. Furthermore, while plasmid copy number is negatively correlated with plasmid size, pAPA3, going against expectations, was retained to a greater degree than the smaller pBR322 and pCAWK (1). Hence, future studies may attempt to insert a non-coding 1.6 kb sequence into pBR322 and pCAWK in order to eliminate the influence of plasmid size when studying the effects of Rop expression on plasmid exclusion.

Furthermore, to verify the results observed in Mo *et al.* and better integrate them with the results of the current study, future studies could include a side by side comparison of pUC19 and pAPA3 co-transformed with pCAWK and pBR322 in order to better understand the effect of the RNA II point mutation in plasmid maintenance and exclusion. Alternatively, a pAPA3-derivative with an RNA II point mutation could be created and used to similar effect. This would allow for the examination of the RNA II point mutation, especially considering that previous studies have shown that it reduces the binding affinity of Rop to the RNA I:RNA II hybrid (10).

To further elucidate the effect of Rop expression on downregulating pAPA3 and pBR322 copy numbers, qPCR can be used to measure the relative amount of DNA for each pAPA3, pBR322 and pCAWK plasmids, relative to genomic DNA elements. This can be used to better quantitatively study their relative copy numbers, similar to the approach used in Appleby *et al* (17). Unique genetic features on pAPA3 and pBR322/pCAWK, such as the gentamicin resistance cassette and the tetracycline resistance cassette, could be amplified in respect to a single copy reference gene in the *E. coli* genome such as

dxs, and it can be used to more precisely determine plasmid copy numbers for each respective plasmid.

To help determine why pAPA3 had a remarkably high transformation efficiency relative to pBR322 and pCAWK, qPCR can be used to determine the amount of each plasmid within single transformants at different time points after heat shock. This would test whether pAPA3 produces more copies of itself during the recovery phase following heat shock, which is one of the possible reasons for its high transformation efficiency relative to pBR322 and pCAWK.

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