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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

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It has been shown that when *Escherichia coli* DH5α cells are co-transformed with pBR322 and pUC19, pUC19 is retained in the host cell while pBR322 is excluded. A possible explanation this phenomenon is that the Rop protein produced in pBR322, but not in pUC19, has caused pBR322 to have a lower plasmid copy number, leading to the exclusion of pBR322. The purpose of this study was to investigate the role of Rop protein in the exclusion effect of pBR322 by using pCAWK, a pBR322 mutant that lacked functional *rop* gene. The inactivation of *rop* gene in pCAWK was verified using plasmid copy number test. Co-transformation tests of pBR322 with pUC19, pBR322 with pCAWK and pCAWK with pUC19 were done to determine the ability of different plasmids to co-exist in the same host and the ability of each plasmid to be retained in the host when co-existence was not permissible. Based on the results, plasmid copy number did not influence the sustainability and co-existence of plasmids. There were two ways to interpret the result for the co-transformation of pUC19 with pCAWK. The first interpretation suggested that the inability of plasmids to co-exist was due to competition for replication resources after sharing the same origin of replication, whereas the second interpretation suggested that the presence of Rop protein prevented the co-existence of plasmids with functional *rop* gene and plasmids without functional *rop* gene. The factor that decides which plasmids are retained in the host cell when plasmids co-exist was not yet determined.

The *Escherichia coli* plasmid cloning vectors pBR322 and pUC19 are widely used to study inserted genes or synthesize particular proteins (2). However, these two plasmids tend not to co-exist in a host. It has been known that after co-transforming *Escherichia coli* cells with pBR322 and pUC19, pUC19 is sustained while pBR322 is excluded after several generations (3). There are three possible explanations for this phenomenon, First of all, unlike pBR322, pUC19 has a point mutation in RNA II, a single stranded RNA that serves as a primer for the synthesis of leading strand. The presence of this point mutation prevents the hybridization of RNA II and RNA I that would otherwise disrupt the initiation of plasmid replication leading to a lower plasmid copy number (1). In addition, the hybridization of RNA I and RNA II is less stable for cells containing pUC19, because pUC19 lacks *rop* gene that codes for Rop protein, a protein that binds to and stabilizes the hairpin pairs that link RNA II to RNA I (6). pUC19 has a smaller plasmid size in comparison to pBR322, which are 2686bp and 4361bp respectively (1). This difference in plasmid size can affect the plasmid copy number because larger plasmids tend to require a longer time than smaller plasmids to finish each replication cycle (1). The three proposed explanations lead to a common effect: pUC19 yields a higher plasmid copy than pBR322. When present individually in a cell, pUC19 has a plasmid copy number of around 75 per cell at 37^0C and >200 per cell at 42^0C, whereas pBR322 has a plasmid copy number of only around 20 per cell (7, 4). Since pUC19 and pBR322 share the same origin of replication (pMB1 origin of replication), they need to compete for replication resources (5). Therefore, it can be hypothesized that after many generations following the co-transformation of pBR322 and pUC19, pUC19 that has a faster replication rate and a higher plasmid copy number than pBR322 eventually outcompete pBR322 in host cells.

To test this hypothesis, the pBR322-derived mutated plasmid pCAWK was created previously such that its *rop* gene was disrupted via insertional inactivation. The inserted gene was 35 bp in size, containing premature stop codons flanking a 6 bp Eco52I/EagI site. It was ligated into the PvuII restriction site within the *rop* gene of pBR322 to terminate the amino acid elongation before Phe-56 and Gly-57, which are essential for Rop functionality, were incorporated into the growing polypeptide. The Eco52I/EagI site in the DNA insert served as a tool to differentiate pBR322 from pCAWK. pBR322 digested by Eco52I/EagI was expected to yield a band at 4361 bp, whereas digested pCAWK was expected to yield two bands at 3251bp and 1144 bp. Although the creators of pCAWK used gel electrophoresis following restriction digestion to prove that the insertion of gene construct was successful, they had no evidence to show that *rop* gene was indeed inactivated after the DNA insertion (1).

TABLE 1. The transformation efficiency of pCAWK, pBR322 and pUC19.

Strains	Plate count (cfu)	
	Transformation with 4 µg of plasmid DNA	Transformation with 7 µg of plasmid DNA
pCAWK	135	120
pBR322	120	120
pUC19	91	111

The goal of this study was to use pCAWK to study the exclusion effect of pBR322, with the hypothesis that the inactivation of *rop* gene in pCAWK would cause pCAWK to have a higher plasmid copy and sustainability than pBR322, thus preventing the exclusion of pCAWK after co-transforming it with pUC19.

MATERIALS AND METHODS

Strains and Plasmids. Plasmid pCAWK was previously constructed and incorporated into *Escherichia coli* DH5α strains (1). Cultures of Escherichia coli DH5α containing pUC19, pBR322 and pCAWK respectively were obtained from MICB 421 Culture Collection, Department of Microbiology and Immunology, UBC, Vancouver. *E. coli* DH5α cells that contained none of the above three plasmid strains were also obtained from the same source.

Plasmid Isolation. In preparation for co-transformation test, plasmid pUC19, pBR322 and pCAWK was isolated from their host *E. coli* DH5α strains. The three *E. coli* DH5α strains were grown overnight in a 30 ml Luria Bertani (LB) broth with 100 µg/ml ampicillin at 37 ^{0}C, 180 rpm. The turbidity of the cultures was measured at OD_{650} before plasmid isolation. The plasmid DNA from each strain was then isolated by following the Midipreparation protocol for preparation of plasmid DNA by alkaline lysis with SDS. (7). The isolated plasmids were suspended in 100 µl of TE and quantified using Nanodrop 2000c (Thermo Scientific). In order to calculate the plasmid copy number of each pure strain, the amount of DNA isolated was divided by the turbidity of the cells measured before plasmid isolation.

Identification of plasmid. To differentiate pUC19 from pBR322 or pCAWK, HindIII (Fermentas, cat#ER0503) was used to linearize plasmid and avoid the presence of multiple bands caused by difference in DNA topology. After the linearization of plasmids, pCAWK, pBR322 and pUC19 are identified on gel according to their plasmid sizes: 4395 bp, 4361 bp and 2686 bp respectively (1). For each digestion, 1 µg of plasmid DNA was incubated with 2 µl of the provided 10X Buffer R, 1 µl of HindIII restriction enzyme and nuclease free water to give a final volume of 20 µl. The mixtures were incubated for at least 18 hours at 37 ^{0}C. When pBR322 were to be identified from pCAWK, Eco 52I/EagI (Fermentas, cat#ER0331) was used to digest 1 µg of DNA according to the manufacturer's protocol. The digest mixtures with final volume of 20 µl were also incubated overnight for at least 18 hours at 37 ^{0}C. 4 µl of gel loading buffer was then added to the digest mixtures before running the mixture on a 0.5% agarose gel (Amresco, Biotech Grade 3:1, cat# 9012366). One lane contained a 1 µg Lambda DNA/HindIII marker (Fermentas, cat# SM0101). Gels were run for around 2 hours at 120 V and were post-stained in an ethidium bromide bath (0.5 µg/ml) overnight.

Transformation efficiency test. *E. coli* DH5α cells that contained none of the three plasmid strains used in this study were grown in 3 ml of Luria Bertani (LB) broth overnight in at 37 ^{0}C, 150 rpm. The next day, the cell culture volume was increased to 30 ml using LB broth and the cells were grown to OD_{650} of around 0.2 to be used as competent cells. To prepare competent cells, cells were chilled on ice for 5 minutes and centrifuged at 6900 rpm in a chilled IEC #819 rotor for 5 minutes. After centrifugation, supernatant was discarded and 1.5 ml of ice cold 50 mM $CaCl_2$ in Tris was added to the pellet. The competent cells were kept on ice for at least 30 minutes. Meanwhile, 4 µg and 7µg of each strain of plasmid DNA was chilled on ice. Tris buffer was added if necessary to ensure that all DNA samples had the same total volume. When the competent cells were ready to be used, 0.05 ml of chilled competent cells was added to each DNA sample. The mixtures were chilled for 30 minutes and then heat shocked for exactly 2 minutes. 1.0 ml of LB broth with 100 µg/ml ampicillin was added to each mixture and the mixtures were incubated at 37 ^{0}C for an hour. Mixtures were then spread on LB agar plate containing 100 µg/ml of ampicillin and incubated at 37 ^{0}C.

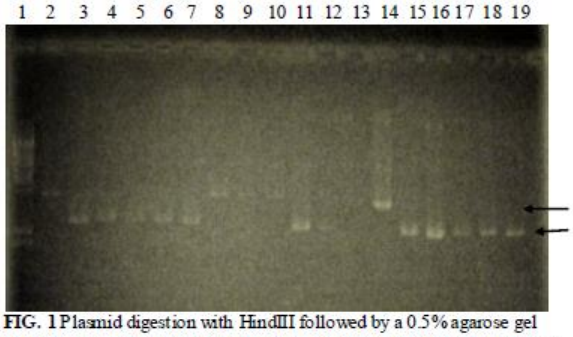

FIG. 1 Plasmid digestion with HindIII followed by a 0.5% agarose gel analysis for plasmid copy number test. Lane 1: λ/HindIII DNA ladder; lane 2: digested pBR322 control; lane 3-18: sample 1 to 16; lane 19: digested pUC19 control; top arrow: pBR322 bands with size of 4361bp; bottom arrow: bands with size of 2686bp.

Co-transformation. The procedure for co-transformation was the same as described in the transformation efficiency test. 7 µg of each plasmid DNA was used for the co-transformation of pUC19 with pBR322 and pUC19 with pCAWK. As for the co-transformation of pBR322 with pCAWK, 4 µg of each plasmid was used.

Plasmid copy number test after co-transformation. After conducting three different co-transformation tests, 16 colonies of co-transformants were isolated from each co-transformation test and grown in 10 ml of Luria Bertani (LB) broth with 100 µg/ml ampicillin overnight at 37 ^{0}C, 180 rpm. The plasmid DNAs of these colonies were isolated as described above. Restriction digestion of the co-transformant DNA was carried out, followed by gel electrophoresis to identify the plasmids that were present in each sample. The plasmid copy numbers of samples that had only one band on the gel were calculated as the amount of isolated plasmids in the sample per OD_{650} of sample cells. When samples showed two bands on the gel due to co-existence of plasmids, the plasmid copy number of each plasmid strain was calculated relative to the estimated intensity ratio of the two bands on the gel.

RESULTS

Plasmid copy number test. The amount of isolated plasmid DNA per the turbidity of cells at OD_{650} was calculated to access the plasmid copy number of each strain. The result showed that pCAWK with 194 µg of DNA per OD of cell had the highest plasmid copy number among the three strains, followed by pBR322 and pUC19, with 122 and 63 µg of DNA per OD of cell respectively.

TABLE 2. Plasmid copy number following co-transformation of pBR322 with pUC19.

Plasmid input ratio (pBR322: pUC19)	Plasmid recovery at each input ratio				Average total plasmid recovery (µg of DNA/ OD650)	Average recovered ratio (pBR322: pUC19)
	pBR322		pUC19			
	Average (µg of DNA/ OD650)	Range (µg of DNA/ OD650)	Average (µg of DNA/ OD650)	Range (µg of DNA/ OD650)		
1:5	8	8	38	38	46	1:5
1:4	5	5	20	20	25	1:4
1:3	2	2	5	5	7	1:3
1:0	64	6-91	0	0	64	64:0
0:1	0	0	53	19-100	53	0:53

This result was unexpected because pUC19 should have a higher plasmid copy number than pBR322. Another problem was that the pUC19 strains used had a very slow growth rate in comparison with the other two strains. Furthermore, the plasmid copy number test also indicated that the *rop* gene in pCAWK was successfully inactivated, due to the fact that pCAWK had a significantly higher copy number than pBR322.

Transformation efficiency test. Besides having a slow growth rate and a low plasmid copy number, the isolated pUC19 plasmids were very difficult to be transformed into *E. coli* DH5α cells. In most of the earlier transformation efficiency tests, the *E. coli* cells that were supposed to be transformed with pUC19 barely had any growth on plates with 100 µg/ml ampicillin, indicating a failure in transformation. After repeating the transformation efficiency test for many times, the following result was finally obtained as shown in Table 1. The transformation using 4 µg and 7 µg of plasmid DNA had similar plate count results (Table 1). This demonstrated that 4 µg was sufficient to ensure that at least one of each plasmid DNA was introduced into *E. coli* cells during co-transformation. This result agreed with the transformation efficiency result obtained by previous MICB 447 students. Nevertheless, 7 µg of plasmid DNA was used to co-transform pUC19 with either pBR322 or pCAWK, considering the low transformation efficiency of the pUC19 used.

Co-transformation. In this study, three different co-transformation tests were conducted: pBR322 with pUC19, pBR322 with pCAWK, and pCAWK with pUC19. In each co-transformation test, the plasmid DNAs from 16 co-transformants were isolated to investigate the sustainability of plasmids and their ability to co-exist, via plasmid copy number test on gel (Fig. 1, 2 and 3). Table 2, 3, 4 and 5 represent the results for the plasmid copy number test.

Among the 16 samples in the co-transformation of pBR322 with pUC19, pUC19 existed individually in eight samples whereas pBR322 existed individually in five samples. There were three samples that showed co-existence of pUC19 and pBR322, with pUC19 having a significantly higher copy number than pBR322 in the same host (Fig. 1 and Table 2). In conclusion, this result was consistent with the result in previous studies in that pUC19 and pBR322 did not usually co-exist and pUC19 had a higher tendency than pBR322 to be retained in the host cells.

In the co-transformation test of pBR322 with pCAWK, seven samples had only pBR322 in the host cells, while five samples had only pCAWK in the host cells. There was only one sample to show co-existence of pBR322 and pCAWK (Fig. 2 and Table 3). In this sample, the two plasmids co-existed in equal plasmid copy number. These results indicated that pBR322 and pCAWK had approximately the same sustainability and they did not tend to co-exist in the same host. Judging from these results, it could be concluded that the inactivation of *rop* gene did not affect the sustainability of the plasmids. However, plasmids without functional *rop* gene could not co-exist with plasmids that contained functional *rop* gene. The quality of results for gel electrophoresis analysis was a problem for the visibility of the bands was low. Lane 7, 12, and 15 had no visible band. The 1144 bp band of pCAWK was also not visible. Due to the quality of the gel, it could be possible that there were lanes with two bands to show co-existence but the other bands were too faint to be seen. Nevertheless, even if co-existence of pBR322 and pCAWK did occur, their plasmid copy numbers were surely not equal.

As shown in the gel result for co-transformation test of pCAWK with pUC19, many lanes contained two bands (Fig. 3). This included lane 2 that contained the digest control pUC19, and samples in lane 3, 4, 5, 6, 7, 12, 13, 14, 15, 16, 18. The two bands in the control pUC19 lane were as the result of topological difference in DNA caused by incomplete digestion. The incomplete digestion in the control pUC19 suggested a possibility of the samples with pUC19 plasmid facing the same problem. To support this possibility, all but one sample lanes that contained a band

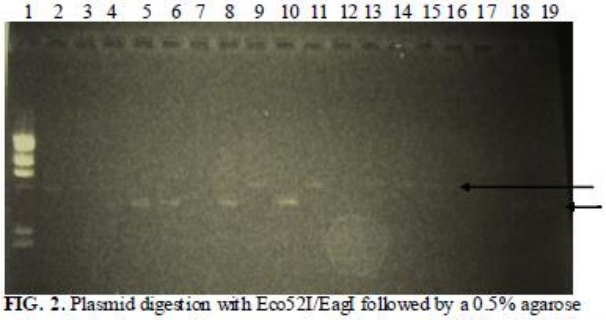

FIG. 2. Plasmid digestion with Eco52I/EagI followed by a 0.5% agarose gel analysis for plasmid copy number test. Lane 1: λ/HindIII DNA ladder; lane 2: digested pBR322 control; lane 3-18: sample 1 to 16; lane 19: digested pCAWK control; top arrow: pBR322 bands with size of 4361 bp; bottom arrow: pCAWK bands with size of 3251 bp.

TABLE 3. Plasmid copy number following co-transformation of pBR322 with pCAWK.

Plasmid input ratio (pBR322: pCAWK)	Plasmid recovery at each input ratio				Average total plasmid recovery (µg of DNA/ OD650)	Average recovered ratio (pBR322: pCAWK)
	pBR322		pCAWK			
	Average (µg of DNA/ OD650)	Range (µg of DNA/ OD650)	Average (µg of DNA/ OD650)	Range (µg of DNA/ OD650)		
1:1	54	54	54	54	108	1:1
1:0	89	25-124	0	0	89	89:0
0:1	0	0	92	74-107	92	0:92

at 2686 bp, which was the size of pUC19, also had another band that was located at the same distance from the gel well as the top band in the incompletely digested pUC19 control. However, the top bands in the samples were only 1 mm lower than the level of digested pCAWK control, representing a difference in around 414 bp in plasmid size. It could be possible that the top bands were pCAWK bands. To support this, it was observed that the intensities of two bands in the sample lanes were mostly equal and did not match the 1:5 intensity ratio of the two bands in pUC19 control lane. The difference in intensity ratio of bands in lane 2 and 3 also proved that the presence of the top band in samples were not a due to cross contamination over wells. Therefore, a major problem for this co-transformation result was that it was difficult to determine whether the top bands of the sample lanes containing pUC19 bands were due to incomplete digestion of pUC19 or co-existence of pUC19 with pCAWK in the same hosts. With the assumption that the top bands were caused by topology difference, the plasmid copy number test showed that eleven samples had only pUC19 plasmids, whereas four samples had only pCAWK plasmid. There was a sample that consisted of both pUC19 and pCAWK, with pCAWK being the dominant plasmid in the host cell (Table 4). This result indicated that pCAWK behaved similarly to pBR322 in that it was also being excluded after co-transformation with pUC19. If the assumption was that the top bands were due to the co-existence of pCAWK and pUC19, pCAWK existed individually in four samples, pUC19 existed individually in only one sample, and they co-existed almost equally in eleven samples (Table 5). It can be concluded from this result that pCAWK and pUC19 had the ability to co-exist at an equally plasmid copy number in the host cell. This result also showed that pCAWK and pUC19 had the same sustainability.

DISCUSSION

According to the results from the plasmid copy number test of isolated pUC19, pBR322 and pCAWK plasmid DNAs (Table 1), insertional inactivation of *rop* gene in pCAWK was successful as expected. However, the obtained plasmid copy number of pUC19 did not make sense, because it should be higher than the copy number of pBR322. Thus, this result did not agree with previous publications (4). The obtained plasmid copy number test result was unexpected because the pUC19 strain used was problematic. It had slow growth rate, low plasmid copy number and low transformation efficiency. Nevertheless, when this pUC19 strain was used in the co-transformation test between pUC19 and pBR322 (Fig. 1 and Table 2), the result obtained was logical and consistent with previous studies. Since the main focus of this study was to investigate the exclusion effect of pBR322 after co-transformation, the problematic pUC19 was usable as long as it did not affect the quality of co-transformation test result. In fact, although the plasmid copy number in the problematic pUC19 was low, the fact that it was more retained in the host cells than pBR322 indicated that plasmid copy number did not affect both the sustainability of plasmids in *E. coli* DH5α cells and the inability of two plasmid strains to co-exist in the same host. It would not be logical for pUC19 that had a very low plasmid copy number and lower plasmid replication to outcompete pBR322 until pBR322 was eliminated. Thus a possible explanation for the inability of pBR322 to co-exist with pUC19 was that since pUC19 and pBR322 shared the same origin of replication, they had to compete for replication resources (5). Although pUC19 with low plasmid copy number had lower replication, it was better than pBR322 at competing for replication resources, leading to the exclusion effect of pBR322.

As for co-transformation test between pBR322 and pCAWK (Fig. 2 and Table 3), the result obtained further supported the previous statement that plasmid copy number did not have a relationship with sustainability of the plasmid. This was because although the plasmid copy number of pCAWK was 1.6X larger than pBR322, the sustainability results of pCAWK and pBR322 were nearly the same. However, these two plasmids tend not to co-exist in the same host cells. Since their only difference was that

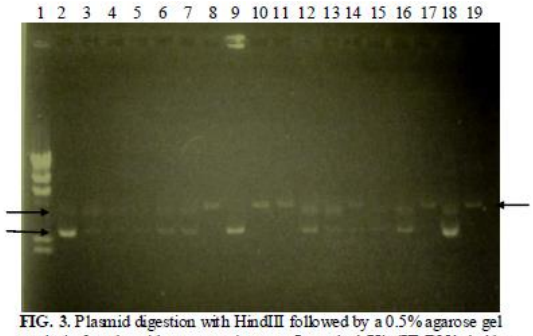

FIG. 3. Plasmid digestion with HindIII followed by a 0.5% agarose gel analysis for plasmid copy number test. Lane 1: λ/HindIII DNA ladder; lane 2: digested pUC19 control; lane 3-18: sample 1 to 16; lane 19: digested pCAWK control; top right arrow: pCAWK bands with size of 4391bp; top left arrow: unknown bands with the size of 3981bp; bottom left arrow: pUC19 bands with size of 2686bp.

TABLE 4. Plasmid copy number following co-transformation of pCAWK with pUC19, assuming that the two bands in the samples were due to incomplete digestion.

	Plasmid recovery at each input ratio				Average total plasmid recovery (µg of DNA/ OD650)	Average recovered ratio (pCAWK: pUC19)
	pCAWK		pUC19			
Plasmid input ratio (pCAWK: pUC19)	Average (µg of DNA/ OD650)	Range (µg of DNA/ OD650)	Average (µg of DNA/ OD650)	Range (µg of DNA/ OD650)		
2:1	50	50	25	25	75	2:1
1:0	81	70-94	0	0	81	81:0
0:1	0	0	44	2-69	44	0:44

pCAWK could not produce functional Rop protein, it could be possible that Rop protein was the factor that determined whether the co-existence of two plasmid strains in the same host cells was permitted. Rop protein regulates plasmid copy number, but plasmid copy number would not have caused the inability of pBR322 to co-exist with pCAWK based on the result obtained from the co-transformation test between pBR322 and pUC19 (Fig.1 and Table 2). This suggested a possible new function of Rop protein in preventing pBR322 from co-existing with other plasmids that did not have a functional *rop* gene. Nevertheless, this explanation did not agree with previous studies on Rop proteins, which showed that the only function of Rop protein was to regulate plasmid copy number by binding RNA complex (1). It was observed that pCAWK and pBR322 were equal in sustainability, so the presence of functional *rop* gene did not decide which plasmid was to be retained in the host cell. Another possible explanation for the inability of pCAWK and pBR322 to co-exist was that the two plasmids shared the same origin of replication and competed for replication resources as previously described. This alternate explanation was consistent with previous publication (5). A problem for this co-transformation test was that the gel results was not very clear, so there may be evidence for co-existence of pBR322 with pCAWK that was not visible. Judging from the plasmid copy number result in Table 3, even if there were co-existence of pBR322 with pCAWK in the samples, their plasmids copy number would not be equal, so the above conclusion would not be affected.

In the co-transformation test between pCAWK and pUC19, there were two ways to interpret the obtained gel results (Fig. 3, Table 4 and Table 5). The first interpretation was the two bands in the sample lanes were resulted from a difference in DNA topology caused by incomplete digestion of samples (Table 4). Another way to interpret the result was the two bands in the sample lanes were caused by the co-existence of pCAWK and pUC19 in those samples (Table 5). Assuming that the first interpretation was right and the samples were incompletely digested, the plasmid copy number test showed that pCAWK behaved exactly like pBR322. This was because both pCAWK and pBR322 could not co-exist with pUC19 and ended up being excluded from most of the samples. This interpretation suggested that the inability of two plasmids to co-exist had nothing to do with Rop protein, because both pUC19 and pCAWK did not have functional *rop* gene. Therefore, co-existence was prevented by competition between plasmids for replication resources, because pUC19 and pCAWK shared the same origin of replication. This explanation for co-existence was supported by previous publication (5). It could be that pUC19 was most capable of competing for replication resources, while pCAWK and pBR322 were equally weak in competing for replication resources. Furthermore, the results in Table 4 also supported the statement that plasmid copy number did not influence the sustainability of plasmids in host cells since pCAWK with the highest plasmid copy number was still excluded by pUC19 that had the lowest plasmid copy number. Besides the capability of competing for replication resources, a possible factor that determined which plasmid strain was to be retained in the host cell would be plasmid size. This was reasonable because pCAWK and pBR322 that had approximately the same plasmid size behaved similarly in the co-transformation test. In fact, when co-transformed, pCAWK and pBR322 were equal in sustainability. On the other hand, pUC19 that had the smallest plasmid size among the three was mostly retained after co-transformation with either pBR322 or pCAWK, because smaller plasmid required less time to complete each replication cycle. Another possible factor that determined the retained plasmid strain was the presence of point mutation in RNA II as in pUC19 (1). When pUC19 was co-transformed with either pBR322 or pCAWK, pUC19 was retained because it had the advantage of having a point mutation in RNAII that prevented the hybridization of RNA I and RNA II that would otherwise slowed down the initiation of replication. In this study, the factor that determined the dominant plasmid strain was yet to be identified. With the assumption that the top bands of the sample lanes in figure 3 were pCAWK bands, the plasmid copy number test result after co-transformation indicated that pCAWK and pUC19 could co-exist almost equally and they shared the same tendency to be sustained in the host cells (Table 5). This result provided support to the statement that *rop* gene was the key that determined the ability of plasmid to co-exist. pBR322 could synthesize Rop protein, which prevented it from co-existing with both pUC19 and pCAWK. In contrast, pUC19 and pCAWK did not have functional *rop* gene, so they had the ability to co-exist in the same host. Although Rop protein prevented the co-existence of plasmid with functional *rop* gene and plasmid without functional *rop* gene, the plasmid that

TABLE 5. Plasmid copy number following co-transformation of pCAWK with pUC19, assuming that the two bands in the samples were due co-existence of both plasmids.

	Plasmid recovery at each input ratio				Average total plasmid recovery (µg of DNA/ OD650)	Average recovered ratio (pCAWK: pUC19)
	pCAWK		pUC19			
Plasmid input ratio (pCAWK: pUC19)	Average (µg of DNA/ OD650)	Range (µg of DNA/ OD650)	Average (µg of DNA/ OD650)	Range (µg of DNA/ OD650)		
1:5	5	5	27	27	32	1:5
2:1	34	18-50	17	9-25	51	2:1
1:2	19	19	38	38	57	1:2
1:1	22	1-35	22	1-35	44	1:1
1:0	81	70-94	0	0	81	81:0
0:1	0	0	61	61	61	0:61

lacked functional *rop* gene was not necessarily the dominant plasmid because pCAWK and pBR322 shared the same sustainability after co-transformation (Fig. 2 and Table 3). As explained previously, plasmid size, point mutation in RNA II or the strength of competing for replication resources could be the factors that determined the dominant plasmid strain. Plasmid copy number was surely not the factor that determined the dominant plasmid strain, because both the pUC19 that had high plasmid copy number as described in previous studies and the problematic pUC19 in this study that had low plasmid copy number could exclude pBR322 after co-transformation.

In conclusion, plasmid copy number did not affect plasmid sustainability and the ability of two plasmids to co-exist. If the two bands in sample lanes in figure 3 were caused by incomplete digestion, the inability of plasmids to co-exist was due to competition for replication resources after sharing the same origin of replication. By assuming that the presence of two bands in sample lanes in figure 3 demonstrated the co-existence of pCAWK and pUC19 in the same samples, it could be concluded that Rop protein had a newly discovered function, which was the ability to prevent the co-existence of plasmid with functional *rop* gene and plasmid without functional *rop* gene. The factor that determines the tendency of the plasmid to be retained in the host cells requires further investigation.

FUTURE DIRECTIONS

The co-transformation test for pCAWK and pUC19 should be repeated but the quality of the result could be improved by increasing the digestion time to over 24 hours. This would avoid incomplete digestion that may cause unclear results. Furthermore, a 2% agarose gel should be used instead during the gel analysis, in order to obtain a higher resolution result and a better separation of the top bands in incompletely digested pUC19 and pCAWK bands.

In order to investigate the statement that plasmid size determines the plasmid strain that is retained in the host cell after co-transformation, the plasmid size of pUC19 could be increased either to the same size as pBR322 or larger than pBR322. This could be done via insertion of reporter genes. The mutant pUC19 could then be co-transformed with either pUC19 or pBR322 to assess its tendency to be retained in *E. coli* cells.

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