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Determination of Exclusion Effects of Potentially *rop* Deficient Mutant pBR322 during Co-transformation with the Wild-type Plasmid

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It has been shown that when pBR322 is co-transformed with pUC19 into the *Escherichia coli* DH5α host bacterial cell, pBR322 is selectively excluded. Furthermore, pBR322 encodes a functional *rop* gene that regulates copy number. The Rop protein is involved in stabilizing the interaction between RNA I and RNA II, which in turn prevents the replication of pBR322. Since pUC19 lacks *rop*, it is of particular interest to investigate whether Rop is responsible for a relatively lower plasmid copy number in pBR322 and the observed selective exclusion. Previously, a mutant pBR322 was created by site-directed mutagenesis such that a mutation was introduced in the ribosome binding site along with a new *Alu* I restriction site. The focus of this study was to determine whether the pBR322 mutant, which should produce less of the Rop protein, will result in a higher copy number and exclude the wild-type plasmid during co-transformation of both plasmids into DH5α. Through extensive gel electrophoresis analysis, it was observed that when mutant and wild-type pBR322 plasmids were co-transformed, the mutant plasmid was selectively excluded from the cell. The observed effect was contrary to expected observation. However, due to time constraints, the results could not be replicated to confirm the probable exclusion effect of mutant pBR322 by the wild-type plasmid.

The major use of plasmid vectors such as pBR322 and pUC19 is to express particular genes and make large amounts of proteins from the inserted genes. Thus, a high plasmid copy number becomes essential for the cloning of recombinant DNA in bacteria. The ability of plasmids to co-exist in a host also allows for the study of their effect upon one another. In the case of pBR322 and pUC19, there are studies which have shown that pBR322 is selectively excluded from the host cell when co-transformed with pUC19 (7).

There are several factors that may play a role in the competitive interaction of pBR322 and pUC19 even though both are derivatives of ColE1-type replicons. The regulation of plasmid replication in pBR322 involves the interaction between specific RNA transcripts, RNA I and RNA II (1). Plasmid replication is initiated by RNA II, which functions as a primer for DNA synthesis as it hybridizes with the DNA template near the replication origin (1). There are two events by which primer formation is inhibited. Firstly, RNA I is complimentary to a section of RNA II and the two transcripts can bind to form an unstable stem-loop complex that inhibits RNA II from initiating replication (2) Secondly, the Rop protein, which is encoded by pBR322, stabilizes the RNA I/RNA II complex and further limits plasmid replication (FIG. 1) (1). Hence, pBR322 is a lower copy plasmid because it is regulated by such protein interactions.

The pUC19 replicates at a higher copy number due to a single point mutation in the RNA II transcript (5). This mutation still allows the interaction between RNA I and RNA II, but the *rop* gene is defective, allowing RNA I and RNA II to dissociate more frequently so there is a greater chance of replication compared to pBR322.

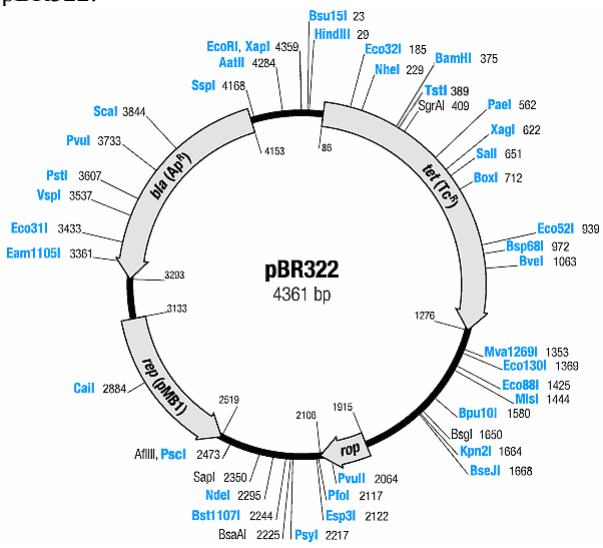

FIG.1 Map of pBR322 showing the location of genes and restriction sites. The *rop* gene and its ribosome binding site are located at positions 1915-2106bp and 1905-1909bp, respectively.

In an attempt to decrease the level of *rop* expression, a mutant pBR322 was created by site-directed mutagenesis such that a mutation was introduced in the ribosome binding site along with a new *Alu* I restriction site (4). Since experimental results from Komljenovic

(4) suggest that the mutagenesis has created a mutation in the ribosome binding site of pBR322, the level of *rop* expression should decrease and thus, the mutant plasmid should have a higher copy number than the wild-type and may result in an exclusion effect between the two plasmids. The wild-type plasmid has 18 *Alu* I restriction sites, and the largest fragment created after digest should be 908bp (4). The mutagenesis contain an extra *Alu* I site, and the largest fragment created after digesting the mutant pBR322 should be 813bp (4). The goals of this study are to confirm the probable identity of the mutant pBR322 by checking the size of restriction fragments generated by *Alu* I digestion and to assess the role of Rop by determining which plasmid, mutant or wild-type, would exclude the other when they are co-transformed into *Escherichia coli* DH5α.

MATERIALS AND METHODS

Plasmid Isolation. To confirm the probable identity of mutant pBR322, cultures of *Escherichia coli* DH5α. (Teaching Lab, Department of Microbiology and Immunology, UBC, Vancouver) containing wild-type pBR322 and cultures of transformed DH5α strains (IK051-IK055, Teaching Lab, Department of Microbiology and Immunology, UBC, Vancouver) containing probable *rop* deficient mutant plasmids were grown at 37°C overnight in six 5 mL Luria Bertani (LB) broth (6) with 100μg/mL ampicillin. The cultures were grown to approximately 1.5 x 10^9 cells per mL and compared to McFarland Standard #5 (cat# 70900). Three milliliters of each sample was then used to isolate and purify plasmids using the PureLink™ HQ Mini Plasmid Purification Kit (Invitrogen, cat# K2100-10) according to the manufacturer's protocol. The purified plasmids were resuspended in 50 μL of sterile water and quantitated using an Ultraspec 3000 (Pharmacia Biotech, Cambridge UK). Subsequent nucleic acid isolations for co-transformations and restriction digestion used the alkaline lysis according to the mini-prep procedure adapted from Sambrook et al. (6). To separate the plasmid from contaminating RNA, the nucleic acid from 10 mL of culture was dissolved in 100 μL of TE containing RNAse (20μg/mL). The samples were then dialyzed in Tris buffer (10mM, pH 7.4) (3).

Restriction Digestion. To determine whether there are differences in digestion patterns between wild-type and mutant pBR322, 0.4 μg of each was digested at 37°C overnight with 1U *Alu* I (Fermentas, cat# ER0011) in a final volume of 25 μL with 10X RE buffer (Fermentas, cat# 9018), water and gel loading buffer (6). The digest mixtures were separated on a 2% agarose gel (Amresco, Biotech Grade 3:1, cat# 9012-36-6). Seven microliters of MassRuler Express DNA ladder mix at 56.5 ng/μL (Fermentas, cat# SM1283) and undigested samples were also loaded onto the gel. Following co-transformations, isolated plasmids (5 μg) were digested at 37°C for 2 hrs and analyzed along with wild-type or mutant pBR322 alone (2 μg, 4 μg) as previously described.

Transformation. Competent *E. coli* DH5α. cells were thawed on ice and 50 μL was used for co-transformation with 2 μg of wild-type and mutant pBR322 in Eppendorf tubes. The mixture was incubated on ice for 30 minutes and heat shocked at 41°C for 1 minute before cooling on ice for 2 minutes. Eight hundred microliters of LB broth (6) was added to the sample and the tube was placed horizontally in a 37°C walk-in incubator on a shaker for 1 hr. Then, the sample was centrifuged at 3000 rpm for 3 minutes and 750 μL of supernatant was discarded. The pellet was re-suspended and 50 μL was spread plated onto MacConkey agar plates (EM Science, Fluorocult™, cat# VL42183) containing 50 μg/mL ampicillin. Colonies were grown in 10 mL LB broth (6) with 100 μg/mL ampicillin and plasmids were isolated and analyzed as described previously.

RESULTS

Identification of mutant plasmid. Probable mutant pBR322 isolated from transformed *E. coli* DH5α cells and wild-type pBR322 were digested with *Alu* I for a 2% agarose gel analysis to determine whether there were differences in digestion patterns between both plasmids. The digestion pattern of the mutant pBR322 was confirmed to differ from the wild-type plasmid by a single restriction fragment (FIG. 2). The top band in lane 3 has an approximate molecular weight of 905bp whereas the top bands in lanes 4 through 7 have an approximate molecular weight of 812bp, consistent with the expected mutant phenotype.

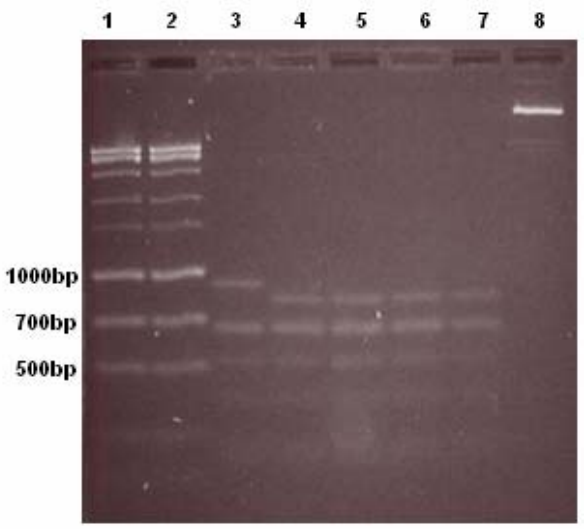

FIG. 2 Plasmid digestion with *Alu* I followed by a 2% agarose gel analysis for identification of mutant pBR322. Lanes 1-2 – MassRuler Express DNA ladder; lane 3 – cut wild-type pBR322; lanes 4-7 – cut mutant pBR322; lane 8 – undigested pBR322. The loaded amounts in each lane were 0.4 μg.

Co-transformation with mutant and wild-type pBR322. Competent *E. coli* DH5α. cells were co-transformed with equal amounts of mutant and wild-type pBR322 and fifty colonies were observed (ET061). Four broth cultures were grown from randomly selected colonies from ET061 and four sets of plasmids were isolated to assess the composition.

Determination of plasmid exclusion effect. The four sets of plasmids isolated from the co-transformation experiment were digested with *Alu* I for a 2% agarose gel analysis to determine whether an exclusion effect has taken place between mutant and wild-type pBR322. Lanes 5-8 revealed wild-type pBR322 banding patterns in the colonies isolated after co-transformation (FIG. 3).

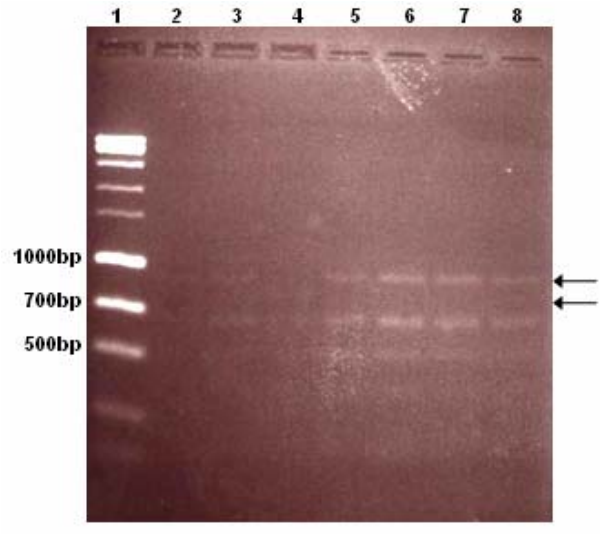

FIG. 3 Plasmid digestion with *Alu* I followed by 2% agarose gel analysis for determination of exclusion effects between mutant and wild-type pBR322. Lane 1– MassRuler Express DNA ladder; lanes 2-3 – 2µg, 4µg cut wild-type pBR322; lane 4 – 2µg cut mutant pBR322; lanes 5-8 – 5µg plasmid sets. The top arrow indicates the top band consistent with the wild-type phenotype in lanes 5-8. The bottom arrow indicates the top band consistent with the expected mutant phenotype in lane 4.

Also, lane 3 has a similar banding pattern as compared to lanes 5-8 with the top band at 907bp. Conversely, the banding pattern in lane 4 with the top band at 815bp was not found in lanes 5 through 8.

DISCUSSION

The plasmids isolated from cultures transformed with IK051-IK055 were identified as mutant pBR322. Since site-directed mutagenesis has created an extra *Alu* I site, the largest band following the mutant plasmid digest should be 813bp (4). As expected, the banding pattern of the mutant plasmid was the same as the wild-type plasmid except for the top band at 812bp which resulted from the incorporation of an additional *Alu* I site (FIG. 1). Similar to findings from Komljenovic (4), the mutant plasmid used for subsequent co-transformation experiments was found to contain a desired mutation in the ribosome binding site as indicated by the presence of an additional *Alu* I site.

The co-transformation experiment suggested that the mutant pBR322 was selectively excluded. The approximate 907bp band characterized by the wild-type plasmid was observed while the approximate 815bp band characterized by the mutant plasmid was not shown in any of the isolated plasmid samples from the co-transformation (FIG. 3). Although some of the bands were difficult to visualize due to the crudeness of the alkaline lysis method for plasmid isolation, the presence of the two potential bands was apparent. Since only four plasmid samples were analyzed from the 50 colonies isolated from the co-transformation, there is the possibility that only the cells that took up the wild-type plasmid were accidentally selected. Nonetheless, the random sampling suggests that the mutant plasmid was in the minority. This effect is contrary to the expectation that the mutant phenotype would exclude the wild-type plasmid and dominate the samples.

In the theory suggested by Komljenovic (4), the level of *rop* expression in the mutant plasmid containing a mutation in the ribosome binding site should decrease, resulting in its higher copy number and possible exclusion of the wild-type plasmid. The findings of this study do not support such a theory because the wild-type plasmid was dominant instead of the mutant. The mutation in the ribosome binding site may have decreased not only the level of *rop* expression but may also have affected other factors essential for maintaining copy number. A closer look at the wild-type plasmid reveals that there does not appear to be readthrough beyond the *rop* gene, so the mutation in the ribosome binding site is not expected to affect other proteins. However, the change could cause sequence effects such that the ability to replicate or sustain high copy number is altered. Therefore, it is important to assess copy number to verify whether the change in the mutant did change copy number. If the change resulted in increased copy number and the mutant plasmid was still excluded, this would suggest that other factors are at work. It may be possible that the wild-type *rop* from the pBR322 might bind in trans to the mutant plasmid and form a more stable complex than the pBR322 complex. In this case, the mutant plasmid might have higher copy number by itself but poorer replication in the presence of the wild-type.

FUTURE EXPERIMENTS

The co-transformation experiment using both mutant and wild-type pBR322 should be repeated to assess that the contrary exclusion effect is real. A larger sampling size should precisely determine whether the two plasmids are capable of co-existing within the same cell.

The results could also be strengthened by measuring the copy number of mutant and wild-type samples to determine whether the mutation causes an increase in copy number similar to that observed for pUC19. This would help verify whether *rop* was actually affected by the mutation in the ribosome binding site and not by the use of readthrough from the *tet* Pribnow box. The method requires maintaining the same concentration

values for all of the cultures grown and calculating the copy number based on the amounts of DNA obtained from gel electrophoresis. Also, the visualization of the DNA bands can be optimized by modifying the alkaline lysis method for plasmid isolation. The plasmid samples should be dialyzed for a longer period of time and with tubing with a higher molecular weight cut-off to facilitate the buffer exchange and to ensure the removal of small contaminants.

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