Journal of Experimental Microbiology and Immunology (JEMI) Vol. 12:118-122

Determination of Exclusion Effect in Wild Type and Rop Deficient Mutated pBR322 Co-transformations

Suzana Sabaiduc and Andy Lo
Microbiology and Immunology University of British Columbia

pBR322 is an expression vector previously reported to be selectively excluded when co-transformed with pUC19 into *Escherichia coli* DH5α. Rop is a regulatory protein encoded on pBR322 but not pUC19. It is involved in stabilizing the RNAI/RNAII complex that inhibits replication of the plasmid. The focus of this study was to determine the role of the Rop protein in the selective exclusion of pBR322 when co-transformed with a mutated pBR322 that is deficient in Rop. This mutation was previously created by site-directed mutagenesis such that a mutation that created a new *Alu I* restriction site was introduced in the presumptive Rop ribosome binding site. When transformed into host cells, mutated pBR322 should produce no Rop protein, therefore resulting in a high mutant copy number. Competent *E. coli* DH5α were co-transformed with equal amounts of wild type and mutated pBR322 to test the relative numbers of transformations. Plasmid DNA was then isolated from the selected transformants, run on an agarose gel, and analyzed for the plasmid band intensities for each plasmid. Our findings showed that when wild type and mutated pBR322 successfully co-transformed into host cells, no exclusion was observed and that doubling the wild type plasmid concentration increased the number of co-transformants by 10 folds. Both findings contradict our hypothesis that Rop is the determining cause of the selective exclusion of pBR322.

The plasmids pBR322 and pUC19 are two commercially available vectors commonly used to express genes of interest. Large amounts of protein are desirable, thus a high plasmid copy number becomes essential when cloning recombinant DNA into bacteria. pUC19 is a pBR322 derivative that replicates at a copy number several fold higher than the parent during growth of *Escherichia coli* (2).

Replication of pUC19 and pBR322 begins with the synthesis of an RNA primer precursor, RNA II (2,4). RNA II first binds and forms a hybrid with its template DNA and is then cleaved at a specific site by the enzyme RNase H, resulting in a free three prime OH end accessible to downstream DNA synthesis by DNA Polymerase I. RNA I is a 108 nucleotide RNA molecule transcribed from a region of the DNA segment encoding RNA II but in the opposite direction. This antisense RNA can bind RNA II and form an unstable stem-loop complex that inhibits RNA II binding to the template DNA, thereby preventing replication of the plasmid (2,4).

It has been observed that when pBR322 and pUC19 are co-transformed, pBR322 is selectively excluded from the host cells. Rop is encoded by pBR322 but not by its pUC19 derivative and is a proposed factor in this selective exclusion of pBR322 since Rop is known to be a negative regulator of plasmid copy number (2,4). Rop was found to accelerate the binding of RNA I to RNA II and stabilize the complex of the complementary RNA I-RNA II stem-loop formation (2,4). Thus, *rop* would prevent the initiation of plasmid DNA synthesis, thereby decreasing plasmid copy number. A three-base substitution mutation using site-directed mutagenesis was previously introduced in the ribosome binding site of *rop* to create a stop codon and an *AluI* restriction site within the *rop* gene (1). This mutant was used to evaluate the potential role of *rop* in the selective exclusion of pBR322, since the mutation should prevent the expression of *rop*.

Wild type *rop*+ pBR322 and mutated *rop*- pBR322 were co-transformed into *E. coli* DH5α to determine whether selective exclusion occurred as a result of the *rop* mutation. If *rop* did play a role, the level of *rop* expression in the mutated plasmid should decrease, resulting in its own higher copy number and possible exclusion of the wild type plasmid. The two plasmids were easily identifiable because wild type and mutant banding patterns following digestion with *Alu I* restriction endonuclease differ in the size of their largest band as a result of the *AluI* restriction site

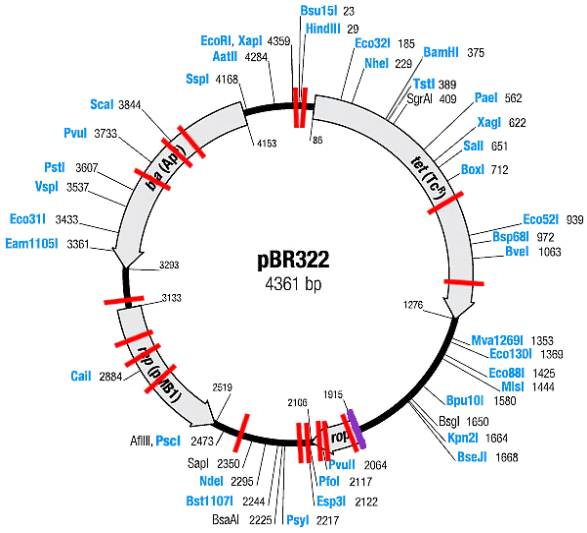

FIG. 1. Map of pBR322 showing the location of genes and restriction sites. The red lines indicate *AluI* cut sites in the wild type pBR322 and the purple line indicates the added *Alu*I restriction site in the mutated pBR322. This additional *AluI* site shortens the wild type 908 bp band to an 813 mutated band.

introduced into the ribosome binding site of *rop* (FIG. 1).

METHODS AND MATERIALS

Strains and Growth Conditions. The wild type plasmid used was pBR322 with the wild type *rop* gene present (MICB 421 Teaching Lab, Department of Microbiology and Immunology, UBC, Vancouver) and the mutated plasmid was pIK051 with a newly generated *AluI* cut site in *rop* (MICB 421 Teaching Lab, Department of Microbiology and Immunology, UBC, Vancouver), likely making it deficient in Rop (1). *E. coli* DH5α (MICB 421 Teaching Lab, Department of Microbiology and Immunology, UBC, Vancouver) was the host bacterium for both plasmids. All *E. coli* cultures containing the plasmids were grown overnight at 37°C in LB media (3) with 100 µg/mL ampicillin (Sigma, A-9518). *E. coli* DH5α cells were made competent by the calcium chloride method according to Sambrook *et al* (3).

Plasmid Isolation. Large-scale isolation of pBR322 and pIK051 was performed to obtain plasmids for transformation. Plasmids were isolated from 500 mL batches using the Invitrogen PureLink HiPure Plasmid Maxiprep Kit (Cat # K2100-07) according to the manufacturer's instructions. The DNA concentration was initially measured using a Biochrom Ultraspec 3000 spectrophotometer in wavelength scan mode from 240-300 nm. However, this method was shown to be unreliable so dilutions of each sample were run alongside dilutions of a pBR322 standard (Fermentas, Cat # SD0061) on a 1% agarose gel and estimates of the actual plasmid concentrations were made by visual comparison after staining in 0.2 µg/mL ethidium bromide.

Transformations. Competent *E. coli* cells were co-transformed using 1 µg of mutated pIK051 plasmid with either 1 µg or 2 µg of wild type pBR322 plasmid according to Sambrook *et al* (3). LB media with 100 µg/mL ampicillin was used during the transformations and cells were plated at 1/10 dilutions onto LB agar plates with 100 µg/mL ampicillin. Plasmid from transformants was isolated by growing select colonies in 5 mL LB media with 100 µg/mL ampicillin overnight and extracting plasmid DNA using Fermentas GeneJET Plasmid Miniprep Kit (Cat # K0502) according the manufacturer's instructions.

Restriction Digest. 10 µL of each isolated plasmid from the transformants was digested overnight at 37ºC with 5U of *AluI* (Fermentas, Cat # ER0011) in 1X Tango Buffer (10X Tango Buffer, Fermentas, Cat # BY5) in a total volume of 50 µL. The restriction enzyme was inactivated at 65ºC for 20 minutes and samples were run on a 2% agarose gel to determine which plasmid(s) were present in each transformed colony selected. Relative copy number ratios for colonies showing both wild type and mutant bands were determined by comparing intensities of the two bands using the program AlphaImager Ver. 4.1.0 Spotdenso application (Alpha Innotech Corp).

RESULTS

Co-transformation with wild type and mutated pBR322. Plasmids from thirty transformed colonies of each wild type to mutant pIK051 ratio combination were isolated, digested with *AluI* and run on a 2% agarose gel (FIG. 2). Both combinations contained colonies with wild type only, mutation only, as well as successfully co-transformed plasmids as summarized in Table I. The ratios of wild type only to mutation only transformants show that transformation efficiency of wild type pBR322 is 1.5 fold greater than that of mutated pBR322, yet does not change with the doubling of wild type pBR322 added to the co-transformation reaction as if the transformation reaction was saturated. However, the percentage of successful co-transformed cells increased by 10 fold with the doubling of wild type pBR322 added.

Relative Plasmid Copy Numbers of Successfully Co-Transformed Cells. Table II describes the relative copy number ratios (as measured from band intensities) of the 11 colonies transformed with both wild type and mutated pBR322. The single successful co-transformed colony in the 1:1 wild type to mutated plasmid ratio has a value of 0.888 while

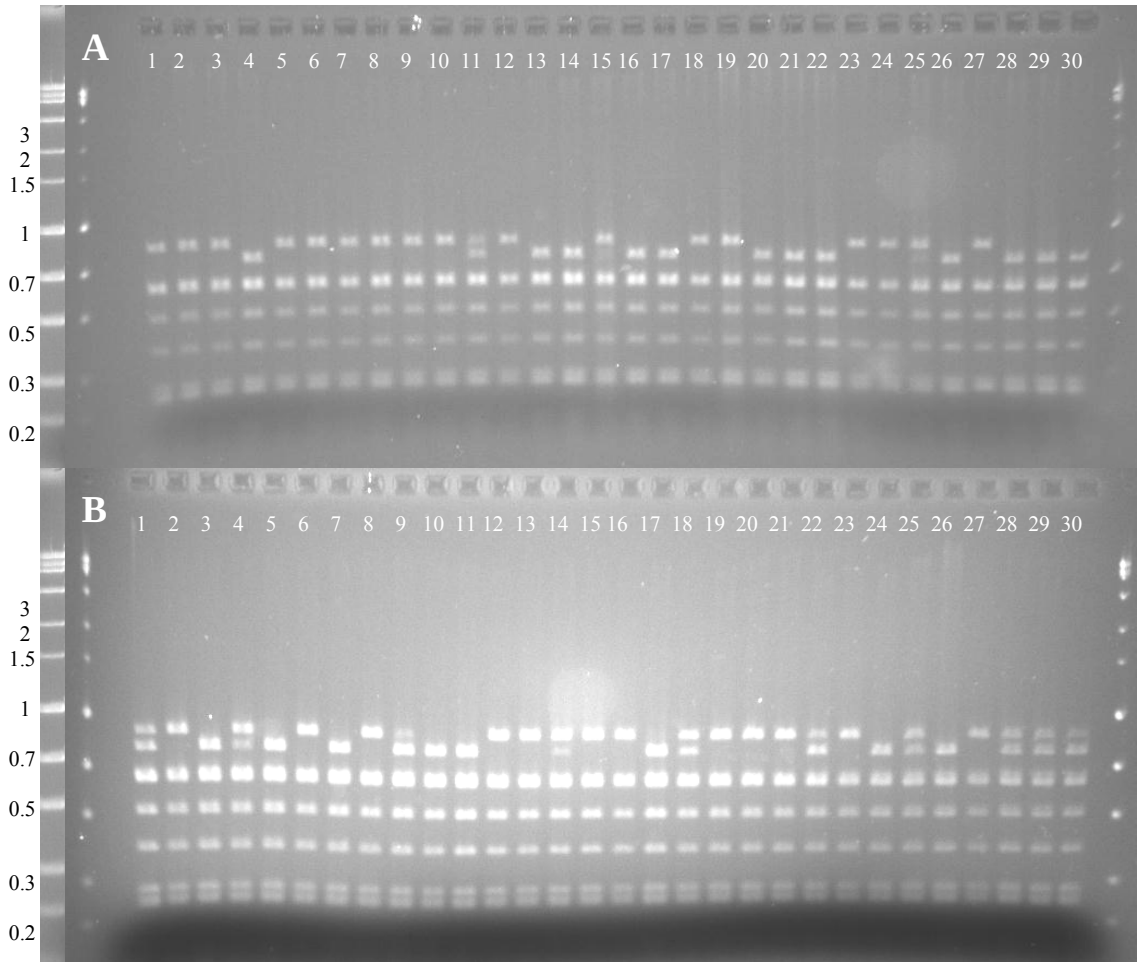

FIG. 2. Isolated plasmid from 60 *E. coli* DH5α transformants from the 1:1 (A) and 2:1 (B) wild type: mutated pBR322 co-transformations digested with *AluI* to differentiate between wild type pBR322 (908 bp band) and mutated pBR322 (813 bp band). Numbers to the left represent ladder (Fermentas, Cat # SM1283) band sizes in kb.

the mean ratio of the ten colonies in the 2:1 ratio is 0.982 with a standard deviation of 0.715.

DISCUSSION

Differentiation between wild type and mutated pBR322 was possible because of the extra *AluI* restriction site mutated into the ribosome binding site of the plasmid with the *rop* mutation. The largest band produced in the digest changed from 908 bp in the wild type plasmid to 813 bp in the mutated plasmid (FIG. 1). After co-transformation of *E. coli* DH5α with the two plasmids, three different types of results were observed on an agarose gel: colonies with a 813 bp band representing transformed cells with only the mutated plasmid; colonies with a 908 bp band representing transformed cells with only the wild type plasmid; and colonies with both bands indicating successful co-transformation with both plasmids. The co-transformations were performed with equal amounts of the two plasmids as well as twice the amount of wild type to mutated plasmid in quantities that would ensure the majority of cells would likely be receiving both plasmids. If the wild type plasmids were to be selectively excluded as was expected, more mutated 813 bp bands should be seen after the *AluI* digest and that number should not increase significantly when the wild type pBR322 added to the co-transformation was doubled. A similar experiment conducted by

Table I. Summary of *E. coli* DH5α results when pBR322 and pIK015 were co-transformed into DH5α.

	Wild type (μg) : Mutation IK051 (μg)	
Ratio of WT : Mutated plasmids	**1:1**	**2:1**
Total number of observed transformants	30	30
WT only transformants	17	12
% of total transformants	57%	40%
Mutation only transformants	12	8
% of total transformants	40%	27%
Ratio of WT only to mutation only transformants	1.43	1.48
WT and Mutation co-transformants	1	10
% of total transformants	3%	33%

Table II. Relative copy number ratios of pBR322 and pIK051 (WT/MUT) in the 11 successfully co-transformed *E. coli* DH5α colonies. Relative copy number ratios were calculated by comparing wild type to mutant band intensity values obtained through AlphaImager's Spotdenso Application (Alpha Innotech Corp). 'Lane' refers to the lanes in numbered in FIG.2; WT IDV refers to wild-type pBR322 intensity density value; MUT IDV refers to mutated pBR322 intensity density value.

1 μg pBR322 : 1 μg pIK051			
Lane	**WT IDV**	**MUT IDV**	**WT/MUT**
11	22365	25185	0.888
		Mean	**0.888**
		Std. Dev.	**N/A**

2 μg pBR322 : 1 μg pIK051			
Lane	**WT IDV**	**MUT IDV**	**WT/MUT**
1	10800	13680	0.789
4	15480	7920	1.955
9	7200	18360	0.392
14	15480	5760	2.688
18	13680	10440	1.310
22	7920	12600	0.629
25	9360	7200	1.300
28	7560	7920	0.955
29	6480	7920	0.818
30	3960	7920	0.500
		Mean	**0.982**
		Std. Dev.	**0.715**

Tsui (5) showed that all four of the colonies sampled had wild type banding patterns, indicating that contrary to previous observations of *rop*+ pBR322 being selectively excluded by *rop*- pUC19, the *rop*- pBR322 was being selectively excluded from co-transformations. However, the sampling size was not sufficiently large to make those conclusions, especially since selection would be slightly biased towards wild type transformants, as was shown in the experiment presented here by wild type pBR322 having 1.5 times greater transformation efficiency compared to mutated pBR322.

The large sample size of 30 transformants chosen in this experiment reflected not only a better representation of all the transformants but also an increased chance of identifying successful co-transformants with both wild type and mutated banding patterns. The fact that some colonies with both plasmids being replicated in the same cell were seen at all (Table II) shows that simply eliminating Rop does not result in selective exclusion of the *rop*+ pBR322. Relative copy number ratios were calculated for the successful co-transformants by comparing the relative band intensities of the 908 bp wild type band against the 813 bp mutated plasmid band. The average ratio of wild type to mutated plasmid intensity in lanes with both bands is 0.982. This would indicate that there is no difference in the copy numbers between the two plasmids when co-transformed into the same cell. Either Rop produced by the wild type plasmid would have the same negative

regulatory effect on the mutated plasmid or the mutation in *rop* was not sufficient to adequately reduce its expression. However, the standard deviation is too large to conclude with certainty that the plasmid copy numbers are the same for the two plasmids when co-transformed into the same cell.

The major limitation in drawing a definite conclusion about the role of Rop in selective exclusion is the inability to determine whether IK051 *E. coli* is truly deficient in Rop. IK051 *E. coli* containing the mutated pBR322 was never proven to be deficient in Rop expression as there is no antibody available to determine protein expression. Had the mutation been sufficient, the copy number of the mutated plasmid should have been higher than the wild type plasmid. The fact that some of the lanes in FIG.2 with individually transformed plasmids showed no difference in their band intensities indicates that the mutation was not sufficient enough to change *rop* expression or activity.

Assuming IK051 *E. coli* with mutated pBR322 is in fact deficient in Rop, our findings indicate that deleting Rop is not sufficient to induce selective exclusion in co-transformations. However, before ruling out the role of Rop in plasmid exclusion, more experiments are required to establish that regulated replication of both plasmids is not due to the negative regulatory effect of the Rop from the wild type plasmid on the mutated plasmid when co-transformed into the same host cell.

FUTURE EXPERIMENTS

Co-transformations of wild type and mutated pBR322 did not cause an exclusion effect on the wild type pBR322. The next set of experiments should focus on comparing co-transformations of wild type pBR322 and pUC19 against co-transformations of mutated pBR322 and pUC19. Wild type pBR322 is expected be selectively excluded in a co-transformation with pUC19. If Rop is responsible for causing this exclusion effect then co-transformation of mutated pBR322 and pUC19 should show transformants that contain both plasmids in quantities comparable to their plasmid copy numbers – mutated pBR322 should not be selectively excluded because there should not be a source or Rop. However, if pUC19 still excludes the mutated pBR322, then this selective exclusion must be caused by a factor or factors other than Rop or the mutation was not sufficient to reduce Rop expression.

Mutations can be targeted to the protein coding region of *rop* to ensure wild type Rop is not expressed, however, if Rop is in fact shown not to be responsible for pBR322 exclusion, then earlier experiments suggesting that plasmid size or length of RNAI transcript (2) could be factors must be revisited.

ACKNOWLEDGMENTS

We would like to thank Dr. William Ramey and Jennifer Sibley for their considerate advise, assistance, resources and technical support throughout this project. We would also like to thank our peers for their help with protocols, sharing of reagents and feedback throughout the duration of the term. Furthermore, we would like to thank the Department of Microbiology and Immunology for providing the financial assistance that made this research possible.

REFERENCES

1. **Komljenovic, I.** 2005. Construction of a mutant pBR322 using site-directed mutagenesis to investigate the exclusion effects of pBR322 during co-transformation with pUC19. J. Exp. Microbiol. Immunol. 8:27-32
2. **Lin-Chao, S., W. T. Chen, and T. T. Wong**. 1992. High copy number of the pUC plasmid results from a Rom/Rop-suppressible point mutation in RNA II. Mol. Microbiol. 6:831-842.
3. **Sambrook, J., Fritsch, E.F. and Maniatis, T**. 2001. *Molecular Cloning: A Laboratory Manual*. 3rd edition, Cold Spring Laboratory, Cold Spring Harbor, NY.
4. **Sozhamannan, S., Morris, J.G. Jr., and Stitt, B.L**. 1999. Instability of pUC19 in Escherichia coli transcription termination factor mutant, rho026. Plasmid. 41: 63-69.
5. **Tsui, E.** 2006. Determination of exclusion effects of potentially *rop* deficient mutant pBR322 during co-transformation with the wild-type plasmid. J. Exp. Microbiol. Immunol. 10:23-26