Journal of Experimental Microbiology and Immunology (JEMI) Vol. 4:39-41

Effect of Altered Plasmid Size on pBR322 Exclusion From pUC19/pBR322 Co-Transformants

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Size modified mutants of the plasmid vector pBR322 were constructed and transfected into *E.coli* DH5∝ cells. After isolating and purifying sufficient amounts of unmodified pBR322, samples were cut using sequential and double-digest restriction enzyme protocols. Cleaving the plasmid with the enzymes *Sty*I and *Msc*I made a small 86 bp deletion, and a larger 295 bp deletion was also made on a separate sample using the *Kpn*2I and *Sty*I enzymes. The cut plasmids were blunt-ended and re-ligated, and transfected into *E. coli* DH5∝ via electro-poration. Clones with the 86 bp deletion were successfully isolated, and the altered plasmids isolated from them. No clones were isolated that contained the 295 bp deletion.

Plasmid vectors are extremely useful tools in molecular biology, allowing production of proteins not normally found within the cell. Depending on the various promoters and regulatory pathways involved in their construction, even the size of the plasmid itself or the chosen origin of replication, the amount of gene product can vary greatly. Smaller plasmids are able to replicate much faster than larger ones, and if unregulated with respect to number, more of the plasmid can fit within the cell. Such 'high copy number' plasmids are useful because more copies of the plasmid per cell means more gene product is produced, and more DNA is available for use in various genetic studies.

Occasionally, it is useful to introduce two different plasmids into a cell, each containing a different gene product, or that utilize different regulatory mechanisms in order to induce protein production under altered conditions. However, cotransfection of two different plasmids is not always successful. The plasmids pBR322 and pUC19, when co-transfected into *E.coli*, experience an exclusion effect where the plasmid pBR322 is consistently lost from the cells while the pUC19 plasmid remains intact. Although these two plasmids are commonly used in a variety of experiments, the precise mechanism by which this exclusion effect is achieved is unknown.

One hypothesis on the cause of the exclusion effect is based off the size of the two plasmids. Because pBr322 and pUC19 both contain the same origin of replication, originally from pMBI (1), the bacterium does not distinguish between the two for purposes of replication. In addition, as the difference in size between the plasmids pUC19 and pBR322 is quite significant, with sizes of 2686 bp and 4361 bp respectively, it is hypothesized that the pUC19 plasmid is retained because of its comparatively small size.

To test this hypothesis, size-modified versions of the pBR322 plasmid were to be constructed, with sizes decreasing to match that of pUC19 such that they could be cotransfected into *E. coli* and the exclusion effect tested. Do to time constraints however; completion of the complete set of size-modified mutants was not possible, and only one 86 bp deletion mutant was successfully generated.

MATERIALS AND METHODS

Reagents, Kits and Enzymes. Nucleotides, restriction enzymes *Sty*I and *Msc*I, BSA and T4 DNA Ligases were from Invitrogen. Ampicillin, tetracycline, Ethanol, Sodium Acetate and LB growth media was from Sigma. Enzyme reaction buffers and T4 DNA Polymerases were from New England Biolabs. The restriction enzyme *Kpn*2I and plasmid purification Mini- and Midi-preps were from Gibco, and the QIAEXII DNA Gel Extraction kit was from Qiagen. The electroporation and gel electrophoresis apparatus were from BioRad.

Bacterial strains. Two *E.coli* HB101 (F-, thi-1, hsdS20 [rB^-,mB^-], recA13, ara-14, leuB6, proA2, lacY1, galK2, rpsL20 [strr], xyl-5, mtl-1, supE44, λ^-) strains, containing the pUC19 and pBR322 plasmids respectively, were used for the initial plasmid production and isolation. Plasmids were over-expressed in both strains through the addition of chloramphenicol (final concentration 0.28 ug/mL) to the cultures upon reaching OD_{660} 0.8, and allowed to continue incubating at 37ºC overnight. A plasmid-free strain of *E.coli* DH5∝ (F-, φ80dlacZ Δ M15, endA1, recA1, hsdR17 (rk^-,mk^+), supE44, thi-1, gyrA96, relA1, Δ(lacZYA-argF)U169, λ^-) was used for the final electroporation and screening. All bacterial cultures were grown in 100 mL batches of LB-Amp media supplemented with 20% glycerol, final ampicillin concentrations of 50 ug/mL.

Plasmid Purification. All plasmid purifications from *E.coli* hosts were performed using the GibcoBRL *Concert High Purity Plasmid Purification System* Midi- and Mini-prep kits. All agarose gel extractions and the purification of plasmid from T4 DNA Polymerase reactions were carried out using the QIAEXII Gel-extraction/DNA purification kit.

Construction of 86 bp and 295 bp deletion mutants. The *Sty*I restriction enzyme, according to the optimal reaction conditions specified by the Invitrogen Catalogue, digested samples of purified pBR322 in 2 hours. The digested DNA was then purified using standard EtOH precipitation

procedures (2) to remove residual reaction buffer incompatible to subsequent enzymes. To generate the 86 bp deletion mutant, a sample of the *Sty*I-linearized plasmid was digested with the *Msc*I restriction enzyme and NEB buffer 3 at 37ºC for 22.5 h. Although not required, BSA was added due to the stabilizing effect it has on endonuclease activity. A separate sample of *Sty*I-linearized plasmid was digested with the *Kpn*2I restriction enzyme to form the 295 bp deletion according to the optimum reaction conditions specified by the GibcoBRL Catalogue.

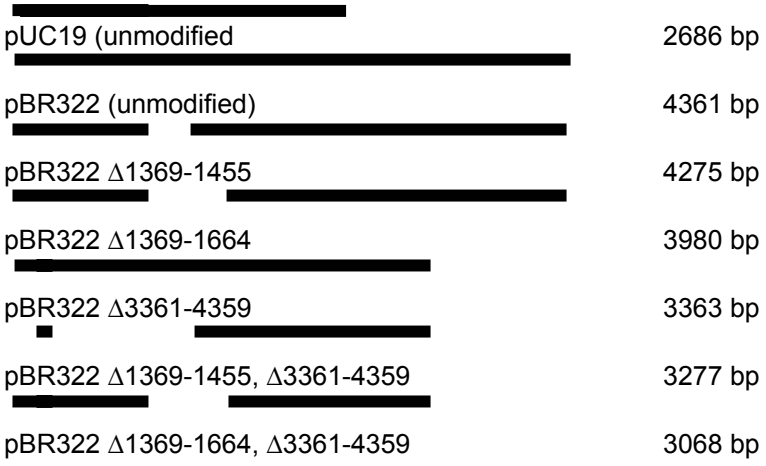

Figure 1. Relative plasmid sizes and modification sites. Shown are the relative sizes and locations of the deletions to be used in preparing the size modified pBR322.

Verification of mutant size. Samples of the altered plasmids were analyzed on 1% agarose gels before ligation to ensure correct cut sites and fragment sizes. Due to the relatively small modification on the 86 bp mutant, the sample was first subjected to additional *Eco*RI digestion to improve resolution.

Recircularization of altered plasmids. The altered plasmids were purified via QIAEXII DNA Purification Kit and incubated at 14ºC for 22 min. in the presence of T4 DNA Polymerase to form blunt ends from the overhanging 5' ends of the DNA fragments. This is slightly different from the normal procedure for T4 DNA Polymerase, which calls for 15 minutes at 11ºC. The products were again purified with the QIAEXII kit to remove the Polymerase, and then incubated at 14ºC for 19 h with T4 DNA Ligase. The results were run through a 1% agarose gel, and the bands matching our target plasmid size were excised and extracted using the QIAEXII Gel Extraction protocol.

Electroporation of *E. coli* DH5∝. Because the concentration of DNA was too low to be accurately determined through spectrophotometry, transfection of the competent *E. coli* DH5∝ cells with the 86 bp mutant was performed twice, once with 15 uL of the ligation product, and once with 5 uL. After electroporation, 100 uL and 500 uL samples of each were used to inoculate an LB-Amp plate before being incubated at 37ºC overnight.

Selection and screening. Half of the colonies seen from the successful transformants were selected at random and used to inoculate 5 mL cultures of LB-Ampicillin media and incubated overnight before being tested for plasmid content with the Gibco Mini-prep kit.

RESULTS AND DISCUSSION

Plasmid purification. The stock plasmids used to prepare the modified pBR322 as well as the pUC19 to test the exclusion effect were analyzed spectrophotometrically for purity and concentration as shown in Table 1 below. The relative purity of the DNA was good, as shown by the ratio of A_{260} to A_{280} values. The amount of DNA isolated from the pBR322 cultures was lower than that of the pUC19 cultures, which can be expected because unlike pBR322, which is regulated to ~20 copies per cell by the Rop gene (1), pUC19 lacks Rop, making it a high copy-number plasmid. However, the chloramphenicol added to the cultures should have removed the normal regulation of pBR322 by Rop, increasing its yield to equal or slightly less than that of pUC19.

Construction of 86 bp and 295 bp deletion mutants. Analysis of the *Sty*I-linearized DNA with agarose-gel electrophoresis resulted in a crisp band migrating at about 4.5 kbp, which is consistent with linearized pBR322. A very faint band running slightly larger than 12 kbp was also visible, and may have been contaminants from genomic DNA (results not shown). Two attempts were made at digesting the *Sty*I-linearized plasmid with the *Kpn*2I enzyme. The first resulted in a faint and ambiguous band, (results not shown) and purification of the preserved band provided insufficient DNA for further use. A second attempt was made, providing much clearer results and sufficient DNA for re-ligation and transfection.

Table 1. Spectrophotometric Analysis of Plasmid Preparations

	OD_{260}	OD_{270}	OD_{280}	260/270	260/280	270/280	[DNA] ug/ul
pBR322	0.804	0.56	0.444	1.226	1.811	1.477	0.402
1/10 dln.	0.666	0.529	0.344	1.259	1.936	1.538	0.333
	0.141	0.119	0.078	1.185	1.808	1.526	0.705
pUC19	0.351	0.289	0.188	1.215	1.867	1.537	1.75
1/100 dln.	0.286	0.237	0.156	1.207	1.833	1.519	1.43
	0.354	0.292	0.193	1.212	1.834	1.513	1.77

A second sample of the *Sty*I-linearized DNA was digested with the *Msc*I restriction enzyme. Because previous attempts to use this particular enzyme had resulted in poor activity, albeit in the presence of TE buffer, which is absent here, we increased the incubation time from 2.5 h to 22.5 h, and added BSA to the reaction mix to help stabilize the digestion. Visualization of the results on a 1% agarose gel with ethidium bromide revealed that the DNA had been slightly over-digested. Despite this over-digestion, the target band was still strongly visible, and appeared to be of the correct size. To confirm this, samples were taken and digested further with *Eco*RI endonucleases, and viewed on a 1% agarose gel. The resulting bands ran around 3000 bp, and 1300 bp in a manner consistent with the deletion (Data not shown). After ligation of the modified plasmids, further analysis after agarose gel electrophoresis implied a successful ligation strategy.

Selection and screening of transformants. The transformation efficiency on all of the electroporated cultures was very low. Only nine colonies were obtained from the 15 uL transfection: one on the 100 uL plate and 8 on the 500 uL plate. The 5 ul transfects fared similarly, with only 3 colonies on the 100 uL plate, and 5 colonies on the 500 uL plate. No clones of the 295 bp mutant were found. During purification of the 5mL cultures prepared from the randomly selected colonies, six of the samples were lost. However, the remaining two samples were observed to contain the plasmid of interest.

This experiment can be thought of as a partial success, for although we did not complete all of our mutants in the time allotted, nor were we even able to compare the one mutant we did obtain, it showed that the materials and techniques used are capable of completing the original experiment, given adequate time.

ACKNOWLEDGEMENTS

This work was supported financially by the Department of Microbiology and Immunology at the University of British Columbia. I would like to acknowledge the invaluable assistance offered by Dr. W. Ramey in implementing and designing our experiment, as well as providing us with the facilities and materials used. I would also like to acknowledge Nick Cheng for all of the help he provided, both in preparing our culture media for use, and for cleanup afterwards.

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