

The Creation of a Size-Modified pUC19

JENNIFER ROGER

Department of Microbiology and Immunology, UBC

In order to study the exclusion effect observed when *Escherichia coli* DH5 α cells are co-transformed with pBR322 (4361 bp) and pUC19 (2686 bp), a modified pUC19 plasmid must be constructed to match the size of pBR322 to determine whether or not it is plasmid size and the resulting stress on the cell during replication and transcription/translation that is the cause of the exclusion. The plasmid will be made by removing, through restriction digests, a 1.7 kb fragment of pBR322, was prepared and purified by gel electrophoresis and extraction. This fragment was isolated and ligated into a pUC19 that was linearized by a restriction digest, using an enzyme with a single recognition site on the plasmid. Constructs were selected for by α -complementation and screened using restriction analysis. None of the isolates were the desired construct.

When the plasmid pBR322 is co-transformed with the plasmid pUC19, pBR322 is excluded from the recombinant host cells (W. D. Ramey, personal communication). This effect was unexpected since pUC19 was derived from pBR322 and has the same origin of replication as pBR322 (1). One hypothesis for this observation is that the size of the plasmid plays a role in determining whether or not it will be excluded; pUC19 is 2686 base pairs and pBR322 is 4361 base pairs. In this experiment, a pUC19 plasmid will be created that matches pBR322 in size for further study of the exclusion effect. A fragment 1.7kb will be removed from pBR322 using restriction endonucleases and inserted into the multiple cloning site (MCS) of pUC19. Mutants will be selected for by screening for the insertional inactivation of the α -complementation shared by pUC19 and its host DH5 α .

The pUC19 plasmid (Fig. 1) will be linearized in the MCS, and a fragment 1.7kb in length will be removed from the pBR322 plasmid (Fig. 2) by restriction digests. The fragment will be isolated and ligated into the linearized pUC19 vector. This mutant plasmid will be electroporated into *E. coli* DH5 α and will be selected for, amplified and purified.

MATERIALS AND METHODS

Plasmids. Plasmids pUC19 and pBR322 were purified from *Escherichia coli* HB101 cultures. Both pUC19 and pBR322 cultures were grown in the presence of 50mg/ml ampicillin until the OD₆₆₀ was 0.677 for pUC19 and 0.792 for pBR322, then incubated overnight in the presence of 100 ug/ml chloramphenicol. The plasmids were purified using GibcoBRL Concert High Purity Plasmid Miniprep System and eluted in water.

Digestions. The pUC19 plasmid (Fig. 1) was linearized with EcoRI, which cuts within the multiple cloning site (MCS), (Invitrogen) in React3 buffer (Invitrogen). The pBR322 plasmid was digested with *EagI* and *PstI* (New England BioLabs) in NEBuffer 3 to remove a 1.7 kb fragment. Other digests included the restriction endonucleases BamHI (Invitrogen) and NdeI (Invitrogen). Digests were run on 1% agarose gels and bands were extracted and purified using Qiagen QIAEX II Agarose Gel Extraction Kit. Sticky ends (on linearized pUC19 plasmid and 1.7 kb fragment) were filled in with T4 polymerase (Invitrogen) according to the supplied protocol. The blunted 1.7 kb pBR322 plasmid fragment was ligated into the linearized, blunted pUC19 plasmid using T4 ligase (Invitrogen) according to the supplied protocol.

Transformation and screening. Electrocompetent DH5 α cells were prepared by following the Invitrogen Zero Background™/Kan Cloning Kit protocol (page 22). The volumes were halved for the entire protocol and in step 1 the culture was spun for 6000 x g for 10 minutes. The competent cells were transformed by electroporation on the *E. coli* setting in 0.2 mm cuvettes.

The DH5 α host contains the mutant gene *lacZ* *DM15* marker which provides α -complementation of the β -galactosidase gene allowing for blue/white colony screening. It also carries *recA1* for increased stability of recombinant inserts by preventing the random recombination of plasmid DNA and *endA1* for improving the quality of the plasmid DNA. The *E. coli* DH5 α genotype information was found on the Biocompare website (<http://www.biocompare.com/itemdetails.asp?List=814,1735>).

The pUC19 plasmid confers ampicillin resistance to its host through its *bla* gene which codes for a β -lactamase that cleaves the β -lactam ring in ampicillin. The pUC19 plasmid also contains the *lacZ* gene which encodes the N-terminal fragment of the enzyme beta-galactosidase. This gene can be induced by IPTG and when synthesized is capable of α -complementation with the defective beta-galactosidase encoded by the host (2). When both gene products are produced, the bacterium is able to break down the sugar X-Gal in the media and as a by-product of the reaction, the colonies turn blue (2). pUC19 contains a multiple cloning site within the *lacZ* gene and so the gene is inactivated when an insert is ligated into the site. Mutant plasmids containing an insert in the MCS will not synthesize a functional N-terminal *lacZ* fragment and so α -complementation will not be possible and the colonies will appear white. pUC19 genotype information was found on the Fermentas website (<http://www.fermentas.com/techinfo/NucleicAcids/mappuc1819.htm>). All cultures were grown in LB broth with 50 ug/ml ampicillin. For selection, 20 mg/ml X-Gal and 0.2 g/ml IPTG was streaked on LB agar plates and dried prior to inoculation with the transformed cells.

RESULTS

The GibcoBRL Concert mini preparations yielded 1.42 ug/ul pUC19 with A_{260} to A_{280} ratios of above 1.8 and 0.402 ug/ul pBR322 with A_{260} to A_{280} ratios of above 1.8 as well.

The purity of the plasmid preparations can be seen in figure 3 where lane 1 is the pUC19 plasmid and lane 2 is the pBR322 plasmid. Lane 3 contains the pUC19 plasmid linearized with *EcoRI* and the size of the plasmid can be verified to be 2.6 kb. Lane 4 contains the pBR322 plasmid from a double digest with *EagI* and *PstI*. The digest yielded two fragments, one of 1.7 kb and the other of 2.6 kb. The 1.7 kb fragment was extracted and purified using the QIAEX II extraction kit and was resuspended in water.

The linearized pUC19 plasmid and 1.7 kb fragment from the pBR322 plasmid were subjected to T4 polymerase reactions to fill in their sticky ends and were ligated together using T4 ligase with 200 ng of vector to 2000 ng of insert. Electrocompetent DH5 α cells were transformed by electroporation with the ligation mix (40 ul cells with 4 ul ligation reaction). The reactions were plated on LB agar plates that had been streaked with 20 mg/ml X-Gal and 0.2 g/ml IPTG.

After 24 hours, both blue and white colonies were visible. Five colonies were picked and grown up in LB ampicillin overnight. The plasmids were purified and digested using *NdeI* and *BamHI* to determine the identity of the plasmids. Digesting with *NdeI* would linearize the pUC19 plasmid thereby allowing for a measurement of its size. Digesting with *BamHI* would result in 2 bands, one restriction site on the pUC19 plasmid and one restriction site on the insert from the pBR322 plasmid. None of the plasmids were the mutant pUC19 plasmid containing the 1.7 kb insert. Each of the plasmids was the wild type pUC19 plasmid indicating that the ligation had failed.

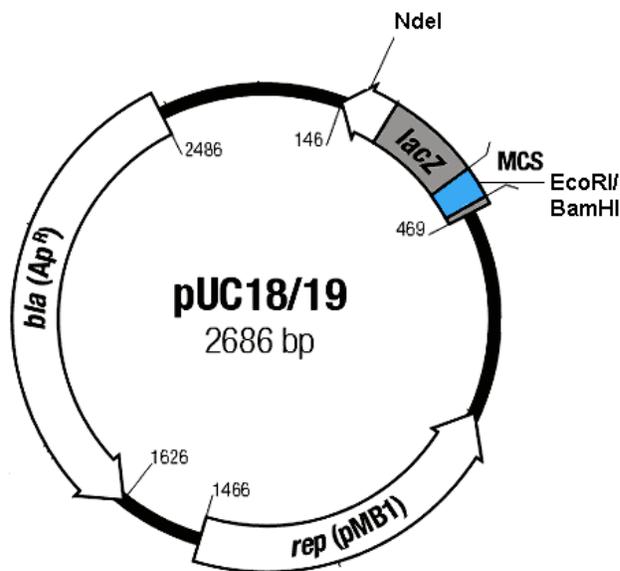


Figure 1. Plasmid map of the pUC19 plasmid. Restriction sites for *EcoRI* (for linearization) and *NdeI*, *BamHI* (for screening) shown. Also shown is *bla* gene for ampicillin resistance, *lacZ* gene for α -complementation and multiple cloning site (MCS).

DISCUSSION

The double digest of the pBR322 plasmid with *EagI* and *PstI* showed a very intense band when it was run on a gel (Fig. 3) that seems to indicate that it was a clean digest with plenty of product. It should be noted that the gel extraction and purification steps lose sufficient quantities of DNA so the amount that was present in the gel was not the amount that was used for the ligations. The bright band at the very bottom of the gel is believed to be talc from the latex gloves that were worn during the loading of the gel.

Since the gel extraction loses quantity of DNA and since the efficiency of blunt end ligations is quite low, no DNA was spared for quantitative studies at each point. It was assumed that half the DNA was lost at each step to allow for a surplus during the ligations. Because the results of the experiment were negative, it would seem that the concentration of insert used in the ligations was too low.

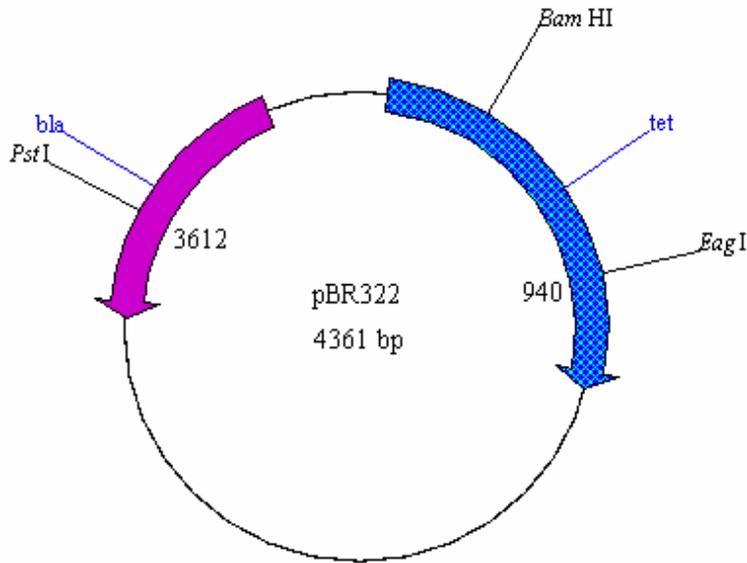


Figure 2. Plasmid map for the pBR322 plasmid. Restriction sites for *Pst*I and *Eag*I show fragment of 1.7kb to be isolated from digest. Also shown is *Bam*HI restriction site for screening as well as *bla* gene for ampicillin resistance and *tet* gene for tetracycline resistance.

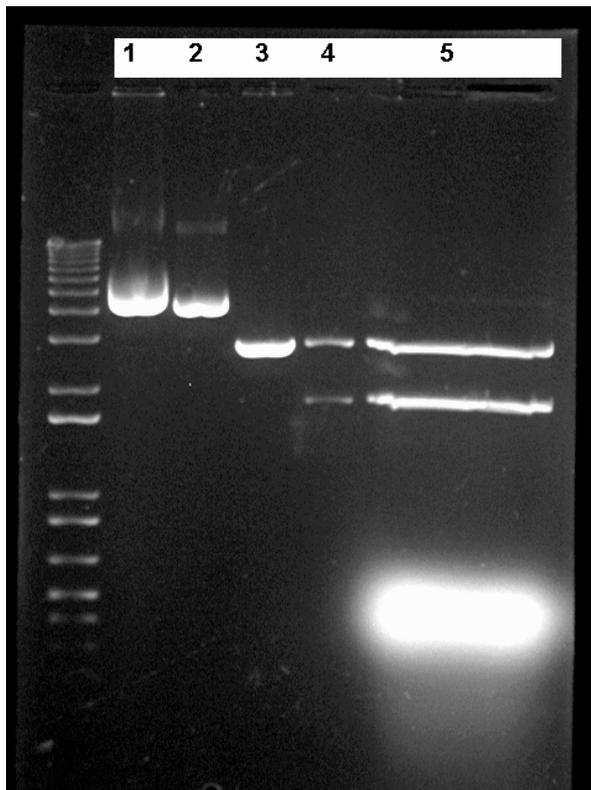


Figure 3. 1% agarose gel containing (1) uncut pUC19, (2) uncut pBR322, (3) pUC19 linearized with *Eco*RI, (4, 5) pBR322 digested with *Eag*I and *Pst*I. The lower band in lanes 4 and 5 are the fragments of 1.7kb that were extracted and purified for ligation into the linearized pUC19 vector.

When screening for the insertional inactivation of β -galactosidase α -complementation, white colonies were found on the plates containing IPTG and X-Gal. This would indicate that β -galactosidase had been disrupted and that the 1.7 kb insert had successfully been ligated into the MCS. However, upon analysis with restriction digests, it was found that the insert was not present. The colonies may have appeared white, not because the insert was present, but perhaps because the IPTG was not spread evenly on the plate and the white colonies were not induced to synthesize β -galactosidase which would then digest the X-Gal to make the blue colour.

Another point of interest was that at one point it was suggested that the stock of pUC19 plasmid might in fact perhaps be pUC19 plasmid mixed with pBR322 plasmid. This was interesting to see because in experiments where the pUC19 plasmid was digested the results appeared normal, but in experiments where the pBR322 plasmid was the target, inconsistent results were obtained which indicated that the stock was not pure.

ACKNOWLEDGEMENTS

I would like to thank Dr. Ramey for his help in designing this project and for clarifying certain elements when they came up. I would also like to thank Nick Cheng in the media room for all of his help autoclaving our supplies and for cleaning up the lab.

REFERENCES

1. **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(22):3385-3393.
2. **Maniatis, T., Fritsch, E.F. and Sambrook, J.** 1991. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
3. **Tang, V., Martin, M., Loutet, S., Lai, J., and Hethey, J.** 2002. Attempts at Cloning pBR322 Δ rop. *Journal of Exp. Microbiol. and Immuno.* 2:90-96.