Journal of Experimental Microbiology and Immunology (JEMI) Vol. 6:67-71

Attempts to Construct a Completely *Rop*$^-$ pUC19 by Using *NdeI* and *EcoO1901* Restriction Endonucleases and Blunt-end Ligation

VIVIENE CHU
Department of Microbiology and Immunology, UBC

Sequence alignments done between cloning vectors pBR322 and pUC19 revealed that pUC19 contained residual 32 bp sequence of the pBR322 *rop* gene, which is responsible for plasmid replication control and thought to play a role in pBR322 exclusion that occurs with co-transformation. Attempts were made to construct a pUC19 plasmid completely without the *rop* gene, by digesting out a segment containing the residual *rop*, and then performing a fill-in reaction and blunt-end ligation to create a *rop*$^-$ pUC19. Efforts at creating such a construct were not successful and *rop*$^-$ pUC19 transformants were not obtained, although positive control results suggest that both the fill-in and ligation reactions were functional. The lack of desired construct could be due to the deletion of a sequence/structure required for replication (e.g. essential region of the H strand Y effector site). Further experiments to test this hypothesis could be done by first inserting pBR322 *rop* gene into pUC19, before removal of the segment containing residual *rop*. Initial attempts at inserting the pBR322 *rop* gene into pUC19 were made during the course of this study. The *rop* gene was amplified from pBR322 template DNA by PCR, and efforts were made to insert the PCR product into an Invitrogen pCR® II-TOPO® Vector. However, results on the identity of potential clones obtained are pending sequencing results. Further studies should include the optimization of fill-in, ligation, and transformation reactions to help increase transformation efficiency.

Plasmid vectors pBR322 and its derivative, pUC19, are well-established multipurpose vectors used to clone and select for recombinant DNA in host bacteria. Both pUC19 and pBR322 are ColE1 derivatives, and are well characterized molecules, with known nucleotide sequences and replication mechanisms. The first step in plasmid replication is the synthesis of an RNA primer precursor (RNAII). RNAII forms a hybrid with the template DNA. Site-specific cleavage of the hybridized RNAII by RNase H creates a 3'-hydroxy, which acts a starting point for DNA synthesis. Replication is controlled by the transcription of a second RNA (RNAI), which is antisense to RNAII. RNAI:RNAII binding inhibits RNAII hybridization with the template DNA, thereby preventing primer formation and replication initiation. Plasmid replication is further regulated by a plasmid-encoded Rop protein, which facilitates and stabilizes RNAI:RNAII interactions, and acts as a negative regulator of replication (5).

pUC19 is a higher copy number plasmid compared to pBR322, and co-transformation of pBR322 and pUC19 leads to pBR322 exclusion. These characteristics might be explained by a number of factors, such as: differences in size (pBR322 is 4361 bp, while pUC19 is 2686 bp), the deletion of the *rop* gene in pUC19, and a point mutation in pUC19 RNAII that prevents RNAI:RNAII interaction (5).

This study will investigate the potential role of the *rop* gene in exclusion and copy number phenotype. Previous work indicated that high copy phenotypes of pUC plasmids are the result of a Rop-suppressible point mutation in RNAII (3), suggesting that Rop plays a key role in copy number regulation. In this study, residual *rop* sequence in the pUC19 plasmid were removed and attempts were made to create a pUC plasmid completely lacking the *rop* gene. It was hypothesized that a *rop*$^-$ pUC19 plasmid should result in a higher copy number phenotype, as Rop has been shown to act as a negative regulator of replication (5). Similarly, a subsequent addition of a *rop* gene into pUC19 should result in a low copy number plasmid, and a possible change in previous co-transformation and exclusion observations. Study results should help direct future experiments and provide a better knowledge of the plasmids' replication and control mechanisms.

MATERIALS AND METHODS

Strains and plasmids. pUC19 and pBR322 were obtained from plasmid preparations on cultures of *Escherichia coli* DH5α (W. Ramey, UBC) containing pBR322 and pUC19, respectively. Cultures were grown in the presence of ampicillin (50 µg/ml) in Luria-Bertani (LB) broth, which was prepared using the following ingredients: 10 g/L tryptone, 5 g/L NaCl, and 5 g/L extract. LB broth was adjusted to a pH of 7 using 1 M NaOH. Cultures were grown to an OD_{660} of approximately 0.75 before the addition and 5 hour incubation with chloramphenicol (0.28 µg/ml), which was used to increase plasmid copy number. Plasmids were isolated and purified using the Qiagen® QIAfilter Plasmid Maxi Kit according to Qiagen® manufacturer protocol. Purified pBR322 and pUC19 were resuspended in Tris-EDTA (TE) buffer.

PCR reactions. The *rop* gene was amplified by PCR on a pBR322 template using the following primers described in table 1.

TABLE 1. PCR primers used in PCR amplification of pBR322 *rop* gene.

Primer	Sequence (5' to 3')*	Restriction Site
Forward	TAT CAT TAC CCC CGG GAA CAG AAA T	*XmaI*
Reverse	GCT GCA GAC GTC AGA GGT TTT C	*PstI*

*restriction site positions underlined in sequence.

Primers were designed using Lasergene DNA and Protein Analysis software (version 6; DNASTAR, Inc., Wisconsin) using the pBR322 sequence (GenBank Accession No. J01749). The PCR reaction mixture consisted of: 2.5 U *Taq* DNA polymerase, 0.3 mM dNTPs, 1X *Taq* buffer, 1.5 mM $MgCl_2$, 0.2 µg pBR322. Total PCR reaction volume was 50 µl. PCR was performed with a Bio-Rad Gene-Cycler PCR machine using the following cycling conditions: an initial denaturation at 94˚C for 3 min, then 40 cycles at 94˚C for 1 min, 55˚C for 1 min, 72˚C for 1 min, followed by a final extension period at 72˚C for 10 min.

Digestions, fill-in reaction, and blunt-ended ligations. Residual *rop* gene sequence was removed from pUC19 by double digestion of the plasmid with *NdeI* and *EcoO1901*. Double digests were performed by incubating the following 50 µl reaction mixture overnight at 37˚C: 5 U *EcoO1901*, 10 U *NdeI*, 1X NEBuffer 4, 100 µg/ml BSA, 5 µg pUC19. The *rop*$^-$ pUC19 digestions were visualized with gel electrophoresis. The *rop*$^-$ pUC19 band was excised, and purified using the Qiagen® MinElute Gel Extraction Kit according to manufacturer protocol. DNA was eluted with TE buffer. Blunt ends were generated by the following fill-in reaction: 0.5 mM dNTPs, 2.8 µg gel-extracted *rop*$^-$ pUC19, 1X REact 2 Buffer, 3 U large fragment DNA Polymerase I, and autoclaved distilled water to 50 µl. Fill-in reactions were incubated at 11˚C, overnight. Reactions were terminated and blunt-ended DNA purified using the Qiagen® MinElute Reaction Cleanup Kit according to manufacturer protocol. Blunt-ended, *rop*$^-$ pUC19 was eluted with TE buffer. Fill-in reaction efficacy was tested by comparing ligation efficiency of *HindIII*-digested λ-DNA fragments with and without a prior fill-in reaction step. Ligation of blunt-ended, *rop*$^-$ pUC19 was done with the following reaction mixture: 1 U T4 DNA ligase, 60 fmol blunt-ended, *rop*$^-$ pUC19 DNA, 1X DNA Ligase Reaction Buffer. Ligation reactions were incubated for 24 hr at 14˚C. Positive controls for fill-in and blunt-ended ligations consisted of reactions using pUC19 DNA digested with *HincII* and *SmaI*, respectively.

Transformation reaction. Two or 5 µl of DNA product from ligation reactions were used to transform *E. coli* DH5α (W. Ramey, UBC). Transformation was done by electroporation of ligated DNA into electrocompetent *E. coli* DH5α, which were prepared using manufacturer protocol. Electroporation was done with a Bio-Rad Micropulser Electroporator using Biorad manufacturer protocol. Electroporated cells were plated on ampicillin-containing (50 µg/ml) LB plates. Colonies potentially positive for the *rop*$^-$ pUC19 plasmid were screened by blue/white screening, plasmid size, and *DrdI* digests (*DrdI* cuts once in *rop*$^-$ pUC19, twice in pUC19). *E. coli* DH5α was transformed with products from each of the following blunt-end ligation reactions: filled-in *rop*$^-$ pUC19, *HincIII*-digested pUC19, and *SmaI*-digested pUC19. A positive control consisted of electroporation of 2 µl of pUC19 DNA into *E. coli* DH5α.

Cloning of pBR322 *rop*. Freshly amplified PCR product (pBR322 *rop* gene) was ligated into the Invitrogen pCR® II-TOPO® Vector, and then transformed into Invitrogen OneShot® Top10 Chemically Competent *E. coli* using manufacturer protocol except that the heat shock was prolonged to 45 sec. Blue/white colony screening allowed for the selection of recombinants, which were selected and cultured in LB broth containing ampicillin (50 µg/ml). Plasmid isolation and purification was done with the Qiagen® QIAfilter Plasmid Maxi Kit according to manufacturer protocol. Purified pBR322 and pUC19 products were each resuspended in TE buffer or autoclaved distilled water.

RESULTS

Amplification of pBR322 *rop*. Each PCR primer was designed to flank the *rop* gene in pBR322, and contained a restriction site to allow subsequent *rop* insertion into the pUC19 multiple cloning site. PCR results were visualized with gel electrophoresis. The expected 247 bp bands were observed, suggesting the successful amplification of the pBR322 *rop* gene. Band excision and gel extraction to obtain purified PCR-amplified pBR322 *rop* was confirmed by gel electrophoresis.

Removal of residual *rop* from pUC19. Figure 1 and Figure 2 show general maps of the plasmids used in this study – pUC19 and pBR322, respectively.

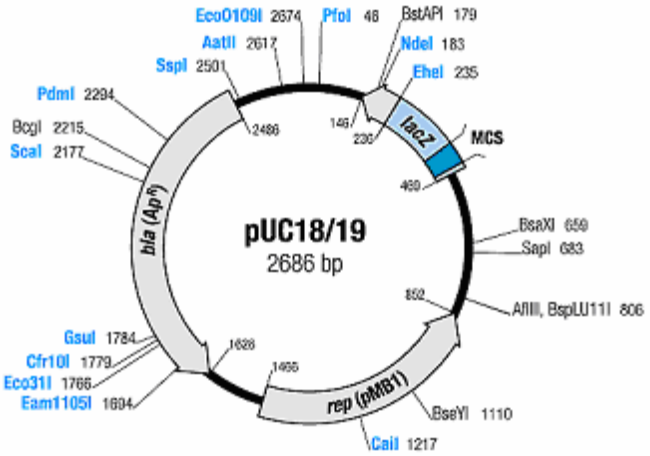

FIG. 1 General map of pUC19 (GenBank Accession No. L09137) (1)

GenBank data on plasmids pUC19 (GenBank Accession No. L09137) and pBR322 (GenBank Accession No. J01749) were used in sequence comparisons. Sequence alignments showed that pUC19 carried a 32 bp sequence corresponding to the 3' portion of the pBR322 *rop* gene. A double digestion with *EcoO1901* and *NdeI* was used to remove a 195 bp segment (which included the residual *rop*), from position 183 to 2674 in pUC19. Specifics on the removed segment are detailed in figure 3.

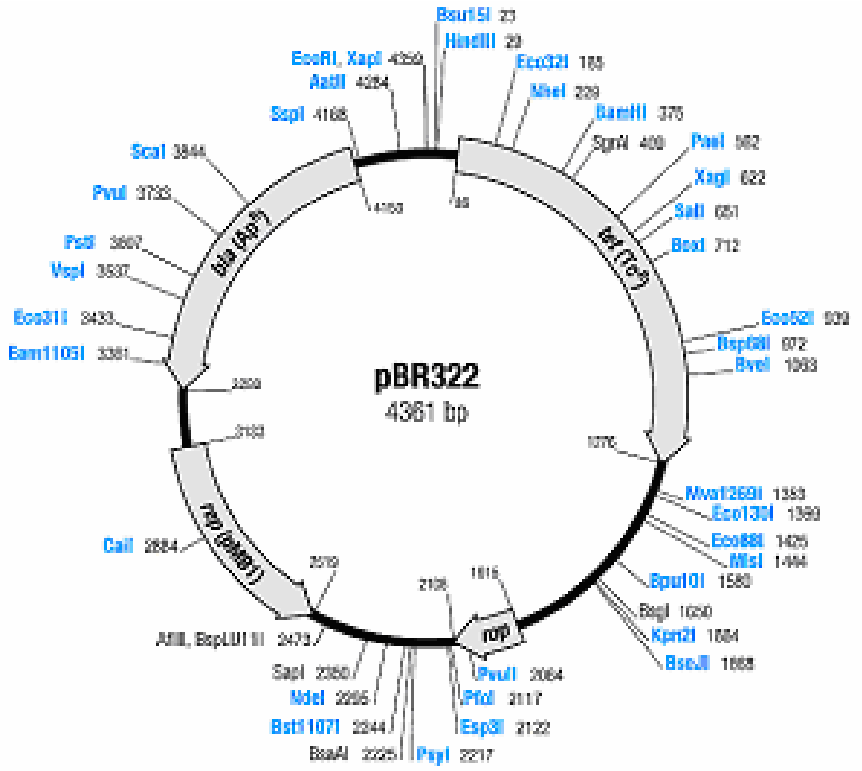

FIG. 2 General map of pBR322 (GenBank Accession No. J01749) (1)

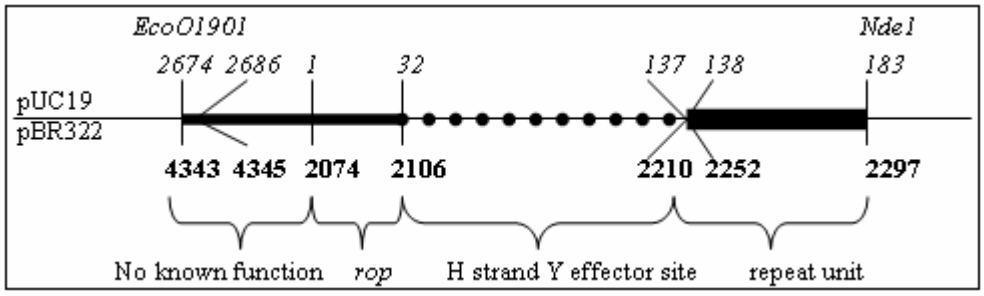

FIG. 3 Details of 195 nt segment removed from pUC19 (from position 2674 to 183). Note that numbers in italics correspond to nucleotide positions in pUC19, and numbers in bold correspond to nucleotide positions in pBR322. Restriction site positions are indicated by the corresponding restriction enzymes.

Successful digestion and removal of residual *rop* in pUC19 was confirmed by gel electrophoresis, which showed the appropriate bands at approximately 200 bp (representing residual *rop*) and 2500 bp (representing pUC19 with *rop* segment digested out).

Fill-in, blunt-end ligation, and transformation of putative *rop*⁻ pUC19

The fill-in reaction efficacy was tested using *HindIII*-digested λ-DNA fragments. Filled-in λ-DNA was observed to have a lower degree of ligation in the ensuing ligation reaction with T4 DNA ligase, suggesting that fragment ends were becoming blunt and more difficult to ligate.

Results of blunt-ended ligation of *rop*⁻ pUC19 DNA were visualized by gel electrophoresis, as seen on figure 4.

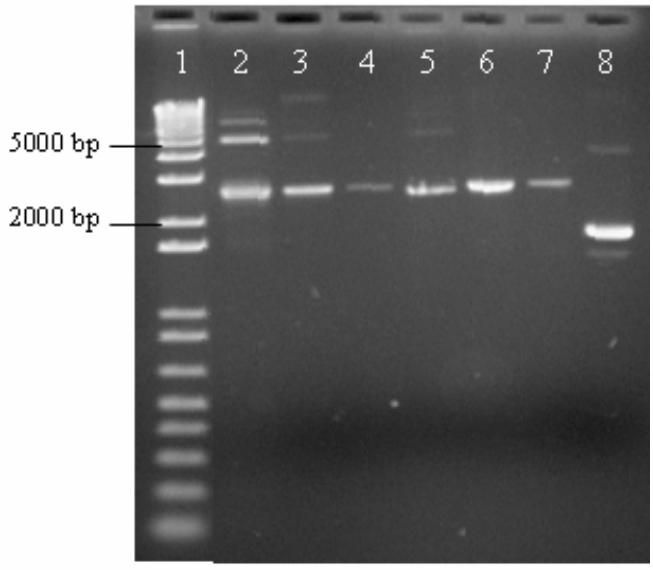

FIG. 4 Gel electrophoresis showing results of blunt-end ligation reactions. Lane 1 – 1 µg 1 kb Plus DNA Ladder; Lane 2 – 0.4 µg *HincIII*-digested pUC19 ligation; Lane 3 – 0.4 µg *SmaI*-digested pUC19 ligation; Lane 4 – spillover from Lane 3; Lane 5 – 0.4 µg filled-in, *rop*$^-$ pUC19 ligation; Lane 6 – positive control, 0.4 µg *HincIII*-digested pUC19; Lane 7 – positive control, 0.4 µg *SmaI*-digested pUC19; Lane 8 – positive control, 0.37 µg uncut pUC19 DNA. Electrophoresis was done using 1.5% agarose gels, run at 120V for 1 hr.

Figure 4 suggests that some blunt-end ligation did occur, as indicated by the presence of bands at positions corresponding to completely closed circular, relaxed, plasmid DNA. However, it is unsure if the bands proposed to represent ligated products were completely closed circular, or if the plasmids were still nicked on one strand.

Ligation reaction products were used to transform *E. coli* DH5α. Results of transformation reactions (table 2) showed that only transformations involving positive controls (restriction enzyme-digested, blunt-end ligations) resulted in colonies, the negative controls showed no colonies for any tested samples.

TABLE 2. Transformation of DH5α *E. coli* with blunt-end ligations of pUC19 DNA.

Ligation Reaction	Transformation Results
HincIII-digested pUC19	Approximately 400 colonies
SmaI-digested pUC19	Approximately 250 colonies
Filled-in *rop*$^-$ pUC19	No colonies

Cloning of pBR322 *rop*. Cloning of pBR322 *rop* into the TOPO® plasmid resulted in 42 potential recombinants (i.e. white colonies). Four of these colonies were chosen at random for plasmid purification and sequencing. Pre-sequencing screening of potential clones was done by double digestion with *XmaI* and *PstI*, which correspond to the restriction sites designed to flank each side of the pBR322 *rop* insert. Digest results, as visualized by gel electrophoresis, showed a single band at approximately 4200 to 4500 bp, suggesting the presence of a singly-digested linearized plasmid. Although this result implies that only one of the expected restriction enzyme sites was present, it was decided to keep the clone for further experimentation. Subsequent plasmid preparation and sequencing steps were performed by Jen Sibley (UBC). Multiple attempts at sequencing plasmid preparation were not successful in yielding sufficient concentrations for sequencing. PCR of the putative *rop* gene resulted in the amplification of a 900 bp product. Sequencing to identify the insert will be done using M13-F and M13-R sequencing primers (found on the TOPO® plasmid). These sequencing results are currently pending.

DISCUSSION

Creation of *rop*$^-$ pUC19 plasmid. The lack of transformants for the *rop*$^-$ pUC19 plasmid could be explained by a number of determining factors at each step of the experiment – from the removal of residual *rop* in pUC19, to the ligation of blunt-ended *rop*$^-$ pUC19, to the transformation of *E. coli* DH5α. For example, when digesting out the residual *rop* sequence from pUC19, other features of the plasmid essential for replication may have been inadvertently removed. Figure 3 details the segment removed, which consisted of: 32 bp of residual *rop*, 12 bp segment of unknown function (according to GenBank information and sequence analysis), 105 bp of an H strand Y effector site, and 45 bp encoding a direct repeat (insertion sequence). The H strand Y effector site deletion may be of particular concern. pBR322 contains regions on opposite strands near the origin which, when single-stranded, act as effectors for *E. coli* replication factor Y, which has ATPase activity (4). Sequence comparisons showed that the H strand effector site in pUC19 was only 53% complete and in a different proximity to the plasmid origin compared to pBR322, and previous work suggested that small deletions would render the site inactive as a factor Y effector (4). Based on these facts, initial thoughts on this deleted region were that it was already inactive, and that pUC19 contained other areas functioning as Y effector sites. The difficulty encountered in obtaining a *rop*$^-$ pUC19 might arise because pUC19 has no other H strand Y effector site, and that the 105 bp in pUC19 contain the essential sequence/structure recognized by factor Y.

Reasons for the lack of transformation of *E. coli* DH5α with putatively ligated *rop*$^-$ pUC19 could be due to a number of variables such as a sub-optimal ligation reaction volume, or the presence of potential inhibitors. However, steps were performed, such as the use of the

Qiagen® MinElute Reaction Cleanup Kit and elution of DNA with TE buffer, to reduce/remove any inhibitory elements in the fill-in reaction such as dATP (a competitive inhibitor of T4 DNA ligase), ion inhibitors (e.g. K^+, Cs^+, NaCl), and EDTA (2). Positive controls of *HincIII*- and *SmaI*-digested pUC19 ligations were successfully transformed in DH5α *E. coli*, indicating that blunt-end ligation and transformation reactions should have worked. Transformations with *HincIII*- and *SmaI*-digested pUC19 ligations resulted in differences in the number of colonies (table 2), with *HincIII*-digested transformants having approximately two times more colonies. This observation could be simply due to natural variation in ligation and transformation efficiency, with the colony numbers representing rough boundaries (i.e. minimum and maximums) as to the expected number of transformants.

Due to the positive control results for the fill-in and ligation reactions, the more probable explanation for the lack of *rop*$^-$ pUC19 transformants would be the deletion of an essential plasmid replication component with the removal of residual *rop*. This could be tested by approaching the construction of *rop*$^-$ pUC19 in a different way – by first *adding* the pBR322 *rop* gene, and then deleting the residual *rop* segment in pUC19. In order to obtain sufficient PCR product of pBR322 *rop* to do ligation experiments, attempts were made to first clone *rop* into a TOPO® vector.

Creation of TOPO® clone containing pBR322 *rop*. Results of cloning the *rop* gene into the TOPO® plasmid strongly support the presence of a 900 bp insert in TOPO®. Further discussion on the insert – its identity, sequence, and implications depends on the sequencing results.

FUTURE EXPERIMENTS

To further test the hypothesis that deletion of the residual *rop* segment in pUC19 also removes a replication-essential component, future experiments should be done where the pBR322 *rop* gene is first inserted into the pUC19 multiple cloning site before residual *rop* is removed from pUC19. Just as a *rop*$^-$ pUC19 phenotype was expected to be a higher copy number, it can be hypothesized that a *rop*$^+$ pUC19 plasmid should lead to a low copy number phenotype and a possible change in the previously observed exclusion phenomena.

Additionally, further attempts could be made to blunt-end ligate and transform *rop*$^-$ pUC19 DNA. The optimal conditions for ligations and transformations (i.e. optimal temperature, incubation time, DNA and salt concentration, addition of PEG8000) could be determined to help increase transformation efficiency. Also, further studies could be done to confirm if the ligations visualized by gel electrophoresis in this experiment represented completely-closed, relaxed plasmids, or plasmids that remained nicked on one strand.

ACKNOWLEDGEMENTS

This work was supported financially by the Department of Microbiology and Immunology at the University of British Columbia. I would also like to thank Dr. W. Ramey for all of the valuable assistance and input throughout the design and implementation of this study. Finally, I would like to express my appreciation to Jen Sibley for her technical assistance and support throughout the term, and for patiently continuing on with plasmid preparations and sequencing of the potential TOPO® clone.

REFERENCES

1. **Fermentas Life Sciences**. 2005. http://www.fermentas.com/techinfo/nucleicacids/mappuc1819.htm; http://www.fermentas.com/techinfo/nucleicacids/mappbr322.htm.
2. **Invitrogen.** 2002. T4 DNA Ligase; Technical Bulletin 15244-2. [Online.]
3. **Lin-Chao, S., W.T. Chen, and T.T. Wong.** 1992. High copy number of the pUC plasmid results from Rom/Rop-suppressible point mutation in RNA II. Mol. Microbiol. **6**:3385-3393.
4. **Marians, K.J., W. Soeller, and S.L. Zipurskey.** 1982. Maximal limits of the *Escherichia coli* replication factor Y effector site sequence in pBR322 DNA. J. Biol. Chem. **257**:5656-5662.
5. **Sozhamannan, S., J. Moris Jr, and B. Stitt.** 1999. Instability of pUC19 in *Escherichia coli* transcription factor mutant, *rho026*. Plasmid. **41:**63-69.