

Attempts at Cloning pBR322Δ(1.1rop)

HEATHER HEINE

Department of Microbiology and Immunology, UBC

The plasmid pBR322 had been observed to be excluded from bacteria also containing pUC19. To investigate whether plasmid size and the presence of the *rop* gene are involved in the exclusion of pBR322 by its successor pUC19, removal of a 1.1kb fragment from the pBR322 vector was attempted to produce pBR322Δ(1.1rop). This alteration reduced the size of pBR322 by 26%, making it closer in size to pUC19. It also removed the *rop* gene, the product of which has been implicated in regulation of DNA replication. Initial preparation of pBR322 and pUC19 DNA from HB101 *E.coli* was successful. Removal of the 1.1 kb fragment with *AflIII* and *StyI* appeared to be successful, however subsequent ligation and transformation of DH5a *E.coli* with pBR322Δ(1.1rop) proved to be challenging. Competent cells were successfully generated and DNA transformation was successful. After restriction digest analysis, however, clones isolated appeared to contain both pBR322 and pBR322Δ(1.1rop). Restriction enzymes employed in this analysis and technical problems with gel electrophoresis posed challenges within the timeline of the study. Thus it is not clear from this work how the reduction in size of pBR322 or the elimination of *rop* expression would affect the exclusion of pBR322 by pUC19.

Replication of ColE1-type plasmids such as the widely used cloning vectors pUC19 and pBR322 begins with the synthesis of the RNA II primer. RNA II must bind with its template DNA and then be cleaved by RNase H to generate a free 3'OH group accessible by DNA Polymerase I (8). RNA I is transcribed in the opposite direction of RNA II, and interaction between this antisense segment and RNA II prevents RNA II from binding to the template DNA, therefore inhibiting DNA synthesis (3, 7, 5, 9-11, 15-17).

In addition to RNA II, replication of pBR322 can also be controlled by the 63 amino acid Rop protein, a product of the *rop* gene. It has been found that deletion of the *rop* gene increases the plasmid copy number approximately two fold (2, 18). Rop has been found to accelerate the binding of RNA I to RNA II and to stabilize the complexes of the complimentary RNA I-RNA II complexes (1, 2, 4, 6, 14, 15). Others have found that the elevated copy number of pUC19 is also due to an *ori* mutation that impedes RNAI:RNAII interaction (13).

It has been observed that *E.coli* transfected with pUC19 and pBR322, both widely used ColE1-type plasmids, eventually cease to contain pBR322 (W. Ramey, personal communication). pBR322 is the parent plasmid of pUC19. Notable differences between the plasmids are that at 4.3 kilobases (kb), pBR322 is 63% larger than pUC19, only pBR322 contains the *rop* gene, and the RNA II in pUC19 has a point mutation that distinguishes it from pBR322 RNA II.

In order to investigate whether plasmid size and the presence of the *rop* gene are involved in the exclusion of pBR322 by pUC19, a 1.1 kb fragment will be removed from the pBR322 vector to produce pBR322Δ(1.1rop). Excision of a 1.1 kb fragment results in removal of approximately 26% of pBR322. This makes the new mutant only 18% larger than pUC19, compared to its original 63%. In addition expression from the *rop* gene will be eliminated. It is hypothesized the combination of these two changes will reduce or eliminate the observed exclusion effect upon co-transfection of pBR322Δ(1.1rop) with pUC19, and will provide direction for future investigation on the precise reason for the exclusion.

METHODS

Strains and plasmids. The bacterial host used as a source for pUC19 and pBR322 was HB101 *E.coli*, a *rec⁻* and *dcm⁻* strain provided by Dr. William Ramey from UBC. The HB101 culture was grown in the presence of 50ug/mL ampicillin to mid log phase, then incubated overnight in the presence of 0.28ug/mL chloramphenicol. Plasmids were purified in columns according to the manufacturer's protocol with the Concert™ High Purity Plasmid Prep System (Invitrogen Canada Inc., Burlington, On.). During this procedure, to improve plasmid recovery the preparation was centrifuged for sixty minutes instead of thirty minutes after its precipitation. As both preparations were completed on different days and by different operators, unintentionally pUC19 was eluted with distilled water, while pBR322 was eluted with TE buffer.

In an attempt to remove a large section of DNA from pBR322, including the *rop* gene, pBR322 was double digested with *AflIII* (to cut the 5' end of the fragment) in combination with *StyI*, *BsmI*, and *AvaI* (to cut the 3' end of the fragment). Three enzymes were used for the 3' end to test restriction enzyme activity (all reagents provided by W. Ramey, UBC). The *AflIII/StyI*, *AflIII/BsmI*, *AflIII/AvaI* double digestions were done in New England Biolabs NEB Buffers 3, 2 and 1, respectively. Unless mentioned all restriction digests were completed for two hours at 37 degrees

Celsius (°C). To generate blunt ends, restriction enzymes were inactivated at 80 °C for twenty minutes before addition of approximately five Units of Klenow Large Fragment (Invitrogen Canada Inc., Burlington, On.) and 0.25mM dNTPs for thirty minutes at room temperature. T4 DNA ligase (Invitrogen Canada Inc., Burlington, On.) was subsequently used to re-ligate the pBR322 fragments for 48 hours at 4°C (on fragments with and without Klenow treatment). Further digestions described include the use of a triple digestion with *PstI/HindIII/EcoRI*, done in REact Buffer 2 (all from reagents from Invitrogen Canada Inc., Burlington, On.). The double digestion of *PstI/EagI* was done in NEB Buffer 3 (all enzymes from Invitrogen Canada Inc., Burlington, On.). Agarose gel electrophoresis was done using 1% agarose gels run at 150 volts. The 1kb Plus DNA Ladder (Invitrogen Canada Inc., Burlington, On.) was employed as a DNA standard. DNA was purified from agarose using QIAEX II Agarose Gel Extraction Kit (Qiagen Inc., Mississauga, On.).

Ligations and Transformations. Competent DH5 α *E.coli* were generated according to the manufacturer's electroporation protocol in the Zero BackgroundTM /Kan Cloning Kit Manual (page 22 of manual, Invitrogen Canada Inc., Burlington, On.). During this protocol DH5 α *E.coli* were grown in 500 mL Luria broth to an OD₆₆₀ of 0.561, and all reagents were reduced by half to account for this reduced volume. Forty microlitres of cells suspended in 10% glycerol were aliquoted into microfuge tubes, quick-frozen in a dry ice/ethanol bath and stored at -80°C until needed for use. Electroporation was done according to the manufacturer's protocol for the Bio-Rad MicropulserTM Electroporation Apparatus (Bio-Rad manual, Bio-Rad Laboratories, Hercules, Ca.) for transformation of DH5 α *E.coli* in 0.2 cm cuvettes (setting Ec2 used). After electroporation, transformed cells were immediately incubated at 37 °C at 225 rpm overnight. Cells were then diluted and spread plated on LB agar and 100 μ g/mL ampicillin. After two days at room temperature, no colonies were visible on any of the plates, including controls. After an additional day incubating at 37 °C, colonies were observed. These were then picked and grown overnight in 3 mL of Luria broth and 100 μ g/mL ampicillin. DNA was mini-prepped in columns according to the manufacturer's protocol of the ConcertTM Rapid Plasmid Purification System (Invitrogen Canada Inc., Burlington, On.).

RESULTS

Removal of 1.1 kb fragment. After maxi-preparation of DNA from HB101 *E.coli*, plasmid concentration and purity was assessed by spectrophotometry (A_{260} , A_{280}) and found to be approximately 380 μ g/mL and 400 μ g/mL for pUC19 and pBR322, respectively. Both plasmids were found to be relatively free from contamination.

Following this, double digestions of *AfIII/StyI*, *AfIII/BsmI*, *AfIII/AvaI* in the appropriate buffer were conducted on pBR322 to facilitate removal of its 1.1 kb sequence. The 5' end of the fragment was cut at 2473 base pairs (bp) with *AfIII*, a restriction endonuclease that generates sticky ends. Between 1369-1426 bp, one of three restriction enzymes was utilized in attempts to cut out the 3' end of the fragment: *StyI* which cuts at 1369 bp, *BsmI* which cuts at 1358 bp, and *AvaI* which cuts at 1426 bp. All enzymes restricting the 5' end also produced sticky ends.

Digestions ran for two hours at 37°C in the appropriate buffer. This was followed by heat inactivation of all restriction enzymes at 80°C for twenty minutes, and introduction of Klenow enzyme for twenty minutes at room temperature. Figure 2 illustrates the results of this digest. Lanes 3-5 appear to show linearized plasmid running at approximately 4.3 kb, as expected for pBR322. Such linearization is characteristic of cleavage at a single site, thus it is unlikely the enzymes were working under these conditions. Lane 6 contains unrestricted DNA, and is faintly visible at under 4.0 kb, likely indicative of supercoiled DNA. Lane 8 appears to show that one *AfIII/StyI* restriction was successful, resulting in approximately 3.2 kb and 1.1 kb fragments expected in this double digestion. Some single cut linearized plasmid at 4.3 kb is also visible indicating the digestion was not complete. Thus *AfIII* and *StyI* were subsequently used to restrict more pBR322 DNA to increase yield of the 3.2 kb fragment for the following experiments.

The second *AfIII/StyI* digestions went for two hours at 37°C in the appropriate buffer. This was followed by heat inactivation of half of the reactions at 80°C and introduction of the Klenow enzyme. Figure 3 illustrates the bands observed by gel electrophoresis.

Lanes 2-7, 9-14 appear to show that the *AfIII/StyI* restriction was successful, resulting in approximately complete digestion of pBR322 into a 3.2 kb fragment. It is believed that the 1.1 kb fragment complimentary to the 3.2 kb fragment, has been restricted out of pBR322 and is obscured by an anomalous substance in the gel (visible between 0.7 – 1.0 kb across the gel). During subsequent electrophoresis, attempts were made to correct the contamination problem, with success only on some occasions. It was assumed the 3.2 kb band contained the correct pBR322 Δ (1.1rop) fragment, and for both the Klenow treated and non-Klenow treated digestions, this band was excised and purified.

Transformation of putative pBR322 Δ (1.1rop). All fragments were ligated and used to transform DH5 α *E.coli* by electroporation. A sample from each treatment of *AfIII/StyI* isolated was used in the transformation, including DNA at low and high concentrations, with and without Klenow treatment. Transformed cells were plated on LB agar containing ampicillin. Colonies grew only in the DH5 α *E.coli* transformed with the higher concentration of DNA, in the mixture that included Klenow treatment. No colonies were observed to grow from any other transformations. The negative controls included cells transformed with no DNA, cells transformed with DNA that was not in contact with DNA ligase, and cells transformed with DNA having contact with neither Klenow nor ligase. None of these transformations resulted in ampicillin resistant colonies as expected. However, the positive control consisting of

DH5 α *E.coli* transformed with circularized pBR322 diluted 1/500 did not produce transformants either. This is likely due to the low concentration of DNA used after the dilution, which would have been 0.8 $\mu\text{g}/\text{mL}$ or 0.004 μg DNA in total.

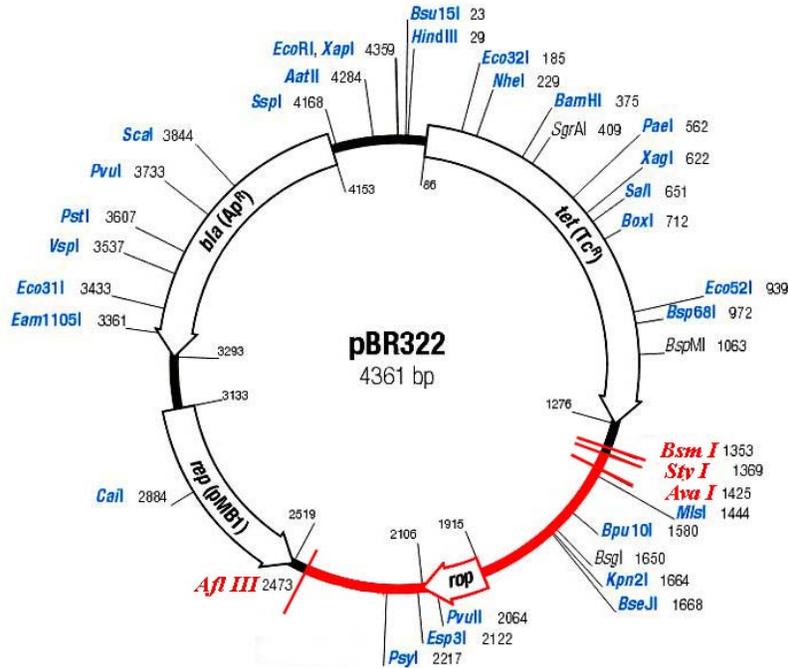


Figure 1. Restriction map of pBR322. Restriction enzyme *AfIII* was used to cut the 5' end, while *BsmI*, *StyI*, and *AvaI* were all attempted to cut the 3' end of a 1.1 kb region removed from pBR322 (shown in red).

Tetracycline and ampicillin resistance cassettes are indicated (*bla* and *tet*, respectively). The *rep* gene represents the *ori* site of the plasmid. Enzymes that cut pBR322 once are shown in blue (12).

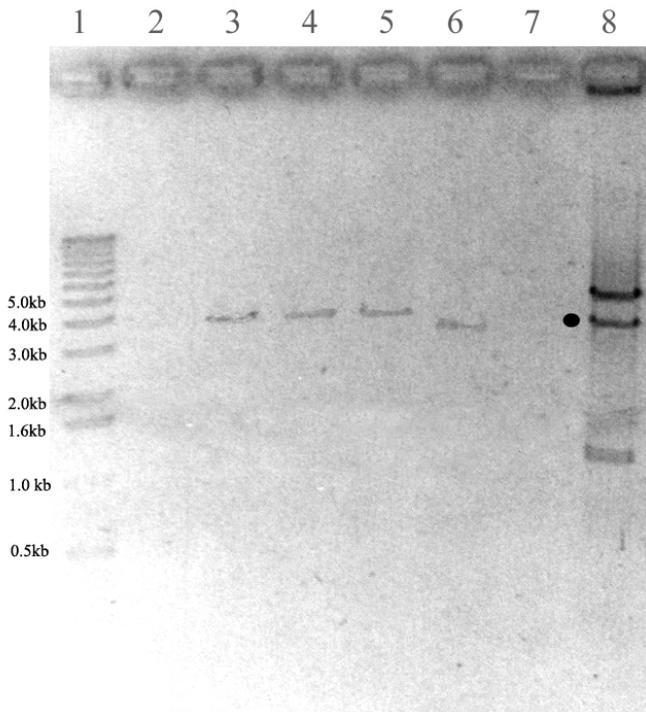


Figure 2. Double Digestion of pBR322.

Lane 1 contains 1kb Plus DNA Ladder. Lane 2 and 7 contain *AfIII/StyI* at a concentration too low for visualization. Lane 3 contains *AfIII/StyI* with DNA at 1/5 dilution. Lane 4 contains *AfIII/BsmI*.

Lane 5 contains *AfIII/AvaI*. Lane 6 contains unrestricted pBR322 DNA. Lane 8 contains more concentrated DNA digested with *AfIII/StyI*. (Note: gel is slightly tilted as part of it broke while acquiring the photograph). The image has been inverted to aid with visualization.

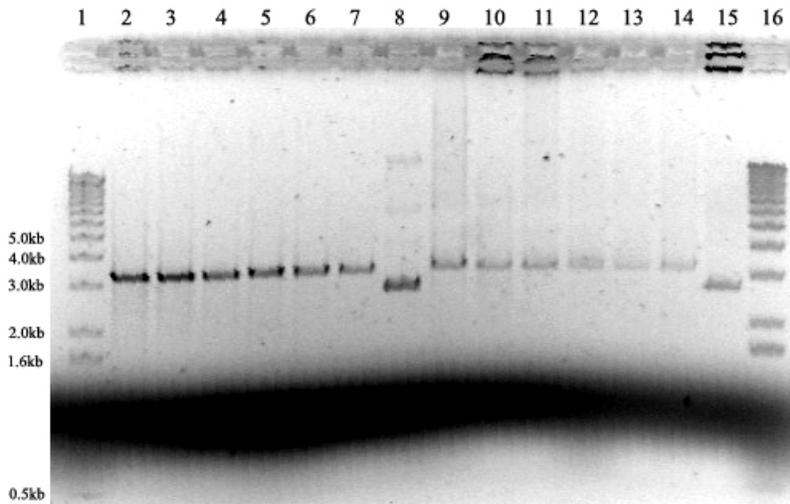


Figure 3. Double Digestion of pBR322 with *AflIII/StyI*. Lanes 1 and 16 are 1kb Plus DNA ladder standards. Lanes 8 and 15 contain unrestricted pUC19 DNA. All other wells contain pBR322 reactions products. Lanes 2-7 contain 6 µL of the *AflIII/StyI* reaction mixture, with lanes 5-7 include Klenow treatment. Lanes 9-14 contain 3 µL of the *AflIII/StyI* reaction mixture, with lanes 12-14 including Klenow treatment. The image has been inverted to aid with visualization.

Analysis of transformed construct. The nineteen colonies that successfully grew on the LB ampicillin agar were subsequently grown up in Luria broth and their DNA was minipreped for analysis. Figure 4 indicates a triple digestion completed on ten of the nineteen samples with *HindIII*, *EcoRI* and *PstI*. *HindIII* and *EcoRI* cut within 31 bp of each other, and were both used to ensure that restriction would be successful, given the uncertain activity of some of the reagents available. Restriction enzyme *PstI* cuts approximately 0.7 kb away from the other two enzymes. Thus if pBR322Δ(1.1rop) had been generated 0.7 kb and 2.4 kb fragments would be expected.

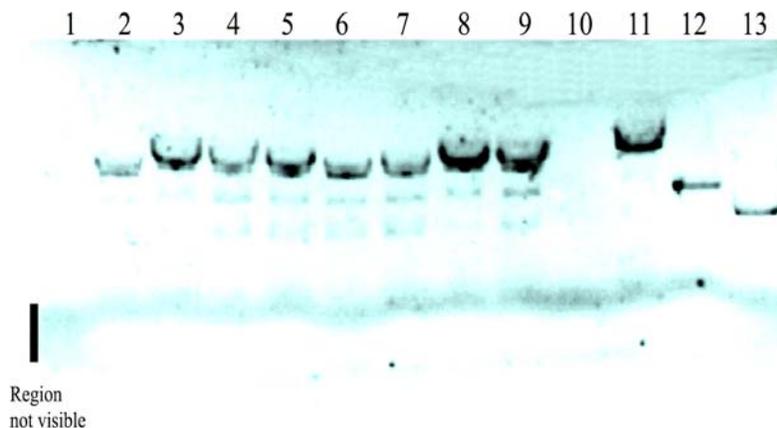


Figure 4. Triple Digestion of Transformed Clones with *HindIII*, *EcoRI* and *PstI*. Lane 1 contained 1kb DNA ladder standard. Lanes 2-11 contain *HindIII*, *EcoRI* and *PstI* triple-digestions of mini-prepped samples (not all samples shown). Lane 12 contains pBR322 also digested with *HindIII*, *EcoRI* and *PstI*. Lane 13 contains supercoiled pBR322. The area obscured by anomalous substance within the gel is indicated. The image has been inverted to aid with visualization.

Unfortunately, none of the three DNA standards in the gel were visible (Fig.4, Lane 1). The anomalous substance in the gel persisted in this experiment, and the region it obscures is indicated. Triple digested pBR322 and undigested supercoiled pBR322 are clearly visible in the gel (Fig.4, Lanes 12 and 13, respectively). From prior

results, including findings of other colleagues, undigested pBR322 runs at approximately 3.6 kb. If the digestion of pBR322 had been successful, fragments would be expected at 3.6 kb and 0.7 kb. As the only clearly visible band in Lane 12 of the gel is larger than the 3.7 kb supercoiled plasmid, it seems likely the digestion was incomplete. The band generated by the digested pBR322 more closely resembles the position of the linearized pBR322 relative to undigested pBR322, as seen in Figure 2 (and other data not shown). The presence of this band in many of clones would also seem to support the suggestion that the reaction was incomplete. Furthermore, by relating the migration distance of these two control samples to their predicted sizes from previous results (see Appendix 1), one can argue that if the reaction was complete, the 2.4 kb fragment would have been 24 millimeters (mm) from the well of the lane, and the 0.7 kb fragment would have been 52 mm from the well (distance not to scale in the figure). This distance puts the 2.4 kb fragment within the obscured region of the gel, and the 0.7 kb fragment off the gel, thus making it unclear whether pBR322 Δ (1.1rop) is present in any of the samples. This hypothesis also does not explain what the larger darker bands are at the top of the wells. It is unclear what they are. If the reaction was incomplete, pBR322 Δ (1.1rop) would have been similarly linearized into a 3.2 kb band. It is possible that the lowest band visible in samples 4-7 and 9, is this 3.2 kb band, when considering its position relative to the postulated 3.6 kb supercoiled pBR322 band in Lane 13 (see Appendix 1, Table A1). Thus, if we assume the reaction was incomplete, it appears linear pBR322 and pBR322 Δ (1.1rop) may both be present in some of the clones.

In order to further the analysis of the isolated clones, another digestion with *Pst*I and *Eag*I was conducted. As Figure 5 indicates, a large area of the gel was again obscured by an unknown substance in the gel. Lanes 2-11 and 14-22 contain the nineteen digested samples. Lane 23 and 24 contain undigested and digested pBR322, respectively. Due to a shortage in remaining plasmid, low concentrations of these controls were used in the hope they would be visible. However, it seems the plasmid concentration was at too low concentration to be seen in these conditions. In this gel it appears the majority of clones again contain linearized 4.3 kb pBR322 plasmid, consistent with an incomplete digestion. If pBR322 Δ (1.1rop) had been present, two approximately 1.6 kb fragments would be expected. No bands of this size are visible in the lower half of the gel, and the upper half is obscured in this region by the anomalous substance, so it is unclear whether they are present. If pBR322 were present, a complete digestion would yield 2.7 and 1.6 kb fragment, however, neither of these are visible.

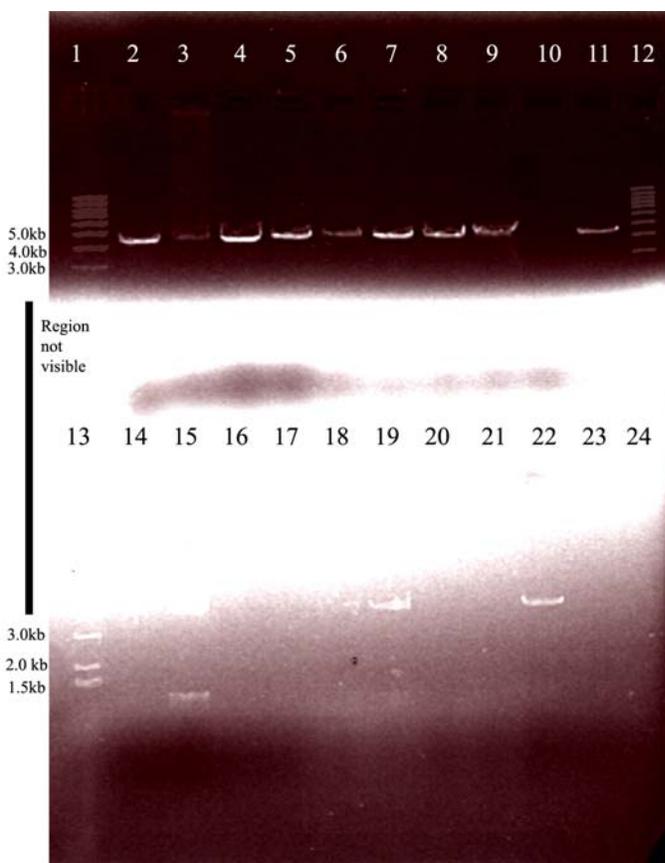


Figure 5. Double Digestion of Transformed Clones with *Pst*I and *Eag*I. Lanes 1, 12, 13, 24 contain 1kb DNA ladder (at different concentrations). Lanes 2-11, 14-22 contain double digested clones. Lane 23 contains uncut pBR322. Lane 24 contains pBR322 cut with *Pst*I and *Eag*I.

Linearized pBR322 Δ (1.1*rop*) and pBR322 would produce 4.3 kb and 3.2 kb bands. The 4.3 kb band is present in most of the clones, suggesting, similar to the gel in Figure 4, one of the restriction enzymes failed to digest the plasmid, and that of the two plasmids, pBR322 is present. It is curious that the 3.2 kb band is not visible in this gel when the same DNA preparations were tested as in the previous digestion.

In a further attempt to improve data acquisition and remove the contaminating substance from the gel, new agarose, new gloves, new buffers and new tubes were used to repeat the above experiment with the remaining DNA. Figure 5 illustrates the results of this digestion. No anomalous substance is visible in any region of the gel. Almost all samples contain a 4.3 kb band characteristic of pBR322, and nearly identical to the *NdeI* linearized pBR322 in Lane 18. The approximately 1.2 kb band visible in many of the wells was unexpected. Lane 19 contains what was believed to be the remainder of supercoiled control pBR322, although the source of this plasmid is questionable, and the band in the gel seems to indicate this sample did not contain pBR322. Lane 17 contains pBR322 digested with *EagI/PstI*, and is the same sample run in Figure 4, Lane 24, but three times more concentrated. The sample contains 2.7 kb and 1.6 kb bands expected of completely digested pBR322. These bands are unexpected if an enzyme in the reaction was not cutting the DNA in the previous experiments. The reasons for this are unclear. This gel appears to support the conclusion that pBR322 was present in the isolated clones. Together, the three digestions seem to indicate that pBR322 was present in many clones, and possibly pBR322 Δ (1.1*rop*).

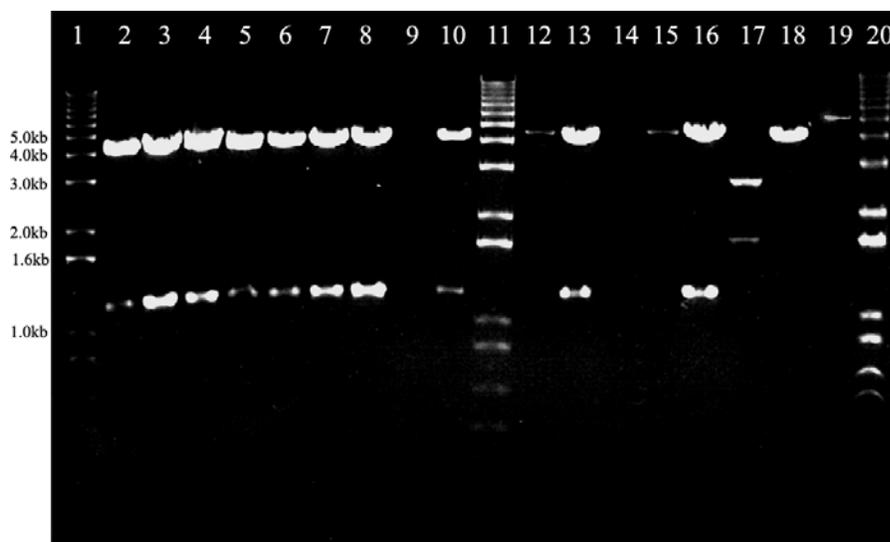


Figure 6. Double Digestion of Clones with *PstI* and *EagI* with New Reagents. Lane 1, 11, 20 are 1kb Plus DNA Ladder. Lane 2-10, 12-16 are double digested samples. Lane 17 is pBR322 cut with *EagI/PstI* (this is the same sample run in Figure 4, Lane 24, but 3X more concentrated). Lane 18 is linear pBR322 cut with *NdeI*. Lane 19 is supercoiled pBR322.

DISCUSSION

To investigate whether plasmid size and the presence of the *rop* gene are involved in the exclusion of pBR322 by pUC19, removal of a 1.1 kb fragment from pBR322 was attempted. pBR322 and pUC19 were successfully isolated from HB101 *E.coli*. Initial trials with available restriction enzymes led to the selection of an *AflIII/StyI* double digest. Enzymes *BsmI* and *PstI* were also tested and digestions including these enzymes were unsuccessful, thus it is possibly that they are not working. Although the fact that only one of four *AflIII/StyI* digestions worked could indicate operator error was also at fault. A large quantity of pBR322 DNA was then restricted with *AflIII* and *StyI* endonucleases and a 3.2 kb fragment was isolated ligated in an attempt to produce pBR322 Δ (1.1*rop*). Of the enzymes tested, the *StyI* digestion was the most ideal as it produces sticky-ends that match in 3 of 4 bases with *AflIII*, improving the chances of sticky-end ligation. The Klenow Fragment DNA Polymerase I enzyme, which functions to fill in 5' overhangs and remove 3' overhangs, was also used to generate blunt ends on the fragment and aid in ligation. This proved successful, as the only surviving *E.coli* transformants were the competent cells electroporated with DNA having Klenow treatment. It was unexpected that the positive control consisting of DH5 α *E.coli* transformed with circularized pBR322 was unsuccessful, however after recalculation, it was realized that after dilution, the quantity of DNA introduced to the cell after was low for effective transformation (Bio-Rad manual, Bio-Rad Laboratories, Hercules, Ca.).

During analysis of isolated clones, various technical problems with digestions, gels and viewing apparatus made the results challenging to interpret. During the first set of digestions with *HindIII*, *EcoRI* and *PstI*, unexpectedly, no DNA standards were visible in the experiment. In order to recover any information from the gel, the results of this digestion needed to be interpreted from controls of pBR322. The hypothesized linearization of pBR322 in the gel not only indicated that one or more of the restriction enzymes used were not working, but somehow pBR322 was present in the isolated clones. If a 4.3 kb band was present, such a fragment could only have been produced from linearized pBR322. Also, based on the extrapolated data, it seems that linear pBR322Δ(1.1rop), at 3.2 kb, was also likely present in many of the same isolates. It is unclear where the large dark band at the top of many of the wells originated from. The presence of these bands in only one of the gels, when the same DNA preparations were used for each digestion experiment suggests that the bands could be products of user error during some part of the electrophoresis procedure. It is possible the agarose gel had not solidified enough prior to creation of the wells, or that DNA contaminants were introduced from the gel buffer. For these reasons, analysis on these clones was continued with more digests.

Additional digestions with *EagI* and *PstI* created similar findings of 4.3 kb bands seem to confirm that pBR322 was present in our isolates, and likely one of these enzymes was not active. In the experiments shown in Figure 5 and 6 however, the 3.2 kb band representing linear pBR322Δ(1.1rop) is not visible. *EagI* and *PstI* both would have cut within the new pBR322Δ(1.1rop), so loss of a restriction site is not likely the problem. It is possible that the exposure time used in the gel made it impossible to see these bands in gels other than the *HindIII*, *EcoRI* and *PstI* triple digestion. During the *HindIII*, *EcoRI* and *PstI* experiment, the faintness of all DNA in the gel, including the standards, likely meant resolving any bands would require a much longer exposure time than any of the other gels. Thus, it is possible that the 3.2 kb bands representing linearized pBR322Δ(1.1rop) were actually present in the gels shown in Figures 5 and 6, but the exposure times during photography may have been too short to detect them.

With regards to the presence of pBR322 in analyzed clones, if we assume for the most part the double digests were incomplete, it appears linear pBR322 and pBR322Δ(1.1rop) may have been transformed into DH5α *E.coli* in some of the clones. The LB agar plates the transformation mixtures were spread on were initially left at room temperature for two days, then following an absence of growth, they were left overnight at 36°C. It is possible that bacteria containing pBR322 may have contaminated the plate while it was being spread, or when it was examined for growth. It is also possible that the transformations were contaminated with DH5α *E.coli* containing pBR322, or some of the bacteria used to generate competent cells contained pBR322, or were contaminated by it.

It is unclear where the 1.2 kb band visible in many clones in Figure 6 and in Lane 15 of Figure 5 originated from. None of the digests attempted were supposed to generate a fragment of this size. It is interesting however, that the distance between the *PstI* restriction site and the *AflIII* site is approximately 1.2kb. It is possible that the ligation of the blunt or sticky ends of *AflIII* and *StyI* generated a restriction site for either *EagI* or *PstI*. All combinations of joining for the two ends do not appear to produce a restriction site for either, but it can not be entirely ruled out. If this were the case however, a corresponding 2.2 kb band of similar intensity should also have been generated, which is not visible in either Figures 5 or 6.

Technical challenges with restriction enzymes and a contaminating substance in the agarose gel also occurred during this study. Once new agarose gel, gel box buffer, loading buffer and gloves were used, the substance was eliminated, at least in the final digestion that was conducted. In hindsight, it would also have been a good idea to run a sample with each enzyme independently to confirm which were working, had enough DNA been available. Ultimately more work is required to continue with the study, however, due to the consumption of all prepared pBR322, this was not feasible in the time allotted. Overall the success with DNA preparation, ligations, competent cell transformation and digestions was better than expected for a short experimental timeline and while working with many untested reagents.

ACKNOWLEDGEMENTS

I would like to thank Dr. William Ramey for his advice, technical support and laboratory resources. Also thanks to Nick Cheng, Jennifer Wong, Jennifer Roger, and Chris Glascock for their support during the course.

REFERENCES

1. **Castagnoli, L., et al.** 1989. Genetic and structural analysis of the ColE1 Rop (Rom) protein. *Embo J.* **8**(2): 621-629.
2. **Cesareni, G., M.A. Muesing, and B. Polisky.** 1982. Control of ColE1 DNA replication: the rop gene product negatively affects transcription from the replication primer promoter. *Proc Natl Acad Sci U S A.* **79**(20): 6313-6317.
3. **Cesareni, G., M. Helmer-Citterich, and L. Castagnoli.** 1991. Control of ColE1 plasmid replication by antisense RNA. *Trends Genet.* **7**(7): 230-235.

4. **Cesareni, G.** 1982. The RNA primer promoter, as defined in vitro, is essential for pMB1 plasmid replication in vivo. *J Mol Biol.* **160**(1): 123-126.
5. **Davison, J.** 1984. Mechanism of control of DNA replication and incompatibility in ColE1-type plasmids--a review. *Gene.* **28**(1): 1-15.
6. **Eguchi, Y. and J. Tomizawa.** 1990. Complex formed by complementary RNA stem-loops and its stabilization by a protein: function of ColE1 Rom protein. *Cell.* **60**(2): 199-209.
7. **Eguchi, Y., T. Itoh, and J. Tomizawa.** 1991. Antisense RNA. *Annu Rev Biochem.* **60**: 631-652.
8. **Itoh, T. and J. Tomizawa.** 1980. Formation of an RNA primer for initiation of replication of ColE1 DNA by ribonuclease H. *Proc Natl Acad Sci U S A.* **77**(5): 2450-2454.
9. **Masukata, H. and J. Tomizawa.** 1990. A mechanism of formation of a persistent hybrid between elongating RNA and template DNA. *Cell.* **62**(2): 331-338.
10. **Masukata, H. and J. Tomizawa.** 1986. Control of primer formation for ColE1 plasmid replication: conformational change of the primer transcript. *Cell.* **44**(1): 125-136.
11. **Polisky, B.** 1988. ColE1 replication control circuitry: sense from antisense. *Cell.* **55**(6): 929-932.
12. Restriction map adapted from
<http://dwb.unl.edu/Teacher/NSF/C08/C08Links/www.fermentas.com/TechInfo/NucleicAcids/mappbr322.htm>
13. **Sozhamannan, S., J. G. Morris and B. L. Stitt.** 1999. Instability of pUC19 in *Escherichia coli* Transcription Termination Factor Mutant, *rho026*. *Plasmid.* **41**(1): 63-69.
14. **Tomizawa, J. and T. Som.** 1984. Control of ColE1 plasmid replication: enhancement of binding of RNA I to the primer transcript by the Rom protein. *Cell.* **38**(3): 871-878.
15. **Tomizawa, J.** 1986. Control of ColE1 plasmid replication: binding of RNA I to RNA II and inhibition of primer formation. *Cell.* **47**(1): 89-97.
16. **Tomizawa, J.** 1984. Control of ColE1 plasmid replication: the process of binding of RNA I to the primer transcript. *Cell.* **38**(3): 861-870.
17. **Wong, E.M. and B. Polisky.** 1985. Alternative conformations of the ColE1 replication primer modulate its interaction with RNA I. *Cell.* **42**(3): 959-966.
18. **Twigg, A.J. and D. Sherratt.** 1980. Trans-complementable copy-number mutants of plasmid ColE1. *Nature.* **283**(5743): p. 216-218.

APPENDIX A – Estimation of Fragment Size in *Hind*III, *Eco*RI and *Pst*I Digestion

The following calculations were used to predict the size of products in the *Hind*III, *Eco*RI and *Pst*I digestion of putative pBR322Δ(1.1rop) clones. If linearized pBR322 and undigested supercoiled pBR322 are expected to run on 1% agarose at 4.3 and 3.7kb, respectively. A two point graph of this relationship is illustrated in Figure A1.

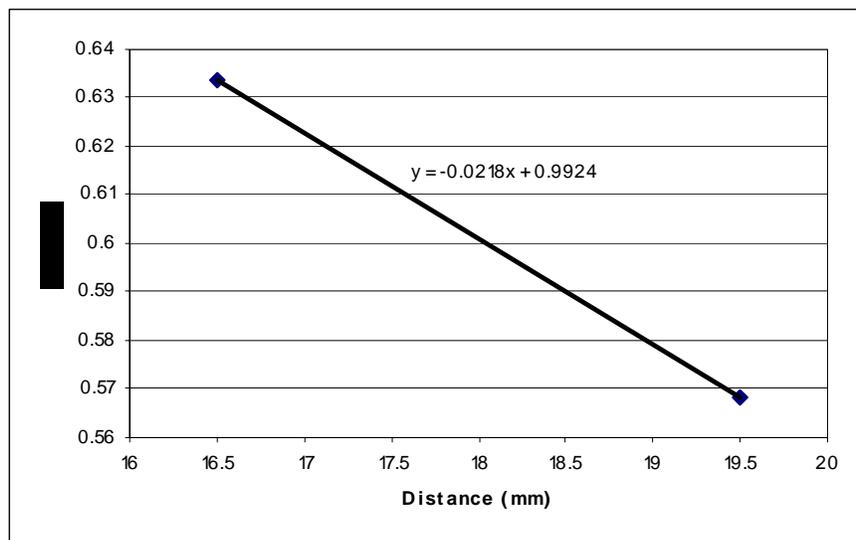


Figure A1. Estimated Relationship Between Migration Distance and DNA Size (kb) in *Hind*III, *Eco*RI and *Pst*I Digest of Putative pBR322Δ(1.1rop) Clones

From this graph and the subsequent relationship

$$[\text{Log (kb)}] = -0.0218[\text{Distance from Well in mm}] + 0.9924$$

the following estimates can be made for other products in a *Hind*III, *Eco*RI and *Pst*I digestion (see Table A1).

Table A1. Estimates of Products in *Hind*III, *Eco*RI and *Pst*I Digestion

Distance from Well (mm)	kb DNA	Fragment
16.5	4.3	Linear pBR322
19.5	3.7	Undigested pBR322
20	3.6	Linear pBR322Δ(1.1rop)
28	2.4	<i>Hind</i> III, <i>Eco</i> RI and <i>Pst</i> I Digested pBR322Δ(1.1rop)
52	0.7	<i>Hind</i> III, <i>Eco</i> RI and <i>Pst</i> I Digested pBR322Δ(1.1rop)