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Construction of an enlarged pUC19 vector with a *rop* gene designed to study plasmid maintenance in *Escherichia coli*

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Experimental evidence has demonstrated that when pUC19 and pBR322 are co-transformed into DH5α *Escherichia coli*, only pUC19 is maintained after a few generations. Possible selective factors include the difference in size between the two plasmids, the presence of a negative regulator *rop* gene encoded by pBR322 and a G→A point mutation in the origin of replication of pUC19. The focus of this study was the construction of a novel enlarged pUC19 vector, pBART, which carries the *rop* and tetracycline resistance genes derived from pBR322. Previous attempts at producing a *rop*$^+$ pUC19 were unsuccessful and failures were attributed to low DNA insert concentration and poor ligation. To circumnavigate these problems, the pBR322 fragment was amplified using PCR and through the process of primer design, a unique XbaI restriction site was created. The PCR fragment was digested and ligated into pUC19 and subsequently transformed into DH5α cells. Disruption of *lacZ* by inserting the pBR322 fragment into the multiple cloning site of pUC19 assisted in isolating transformants carrying the pBR322 fragment. The pBART construct was confirmed by restriction mapping and sequencing. The pUC19 derived pBART construct is comparable in size to pBR322 and can be used to explore how the role of plasmid size, presence of *rop* and the origin of replication G→A point mutation contributes to differences in plasmid copy number during cotransformation of pUC19 and pBR322.

Plasmids pUC19 and pBR322 are common cloning vectors derived from the plasmid pCOlE1 (1). It has been experimentally observed that co-transformation of pBR322 and pUC19 results in the selective maintenance of pUC19 over pBR322 in *Escherichia coli* DH5α cells (2). This observation has been attributed to a point mutation (G→A) in the origin of replication and lack of the *rop* gene in pUC19 (1). In both pUC19 and pBR322, replication is regulated by the RNA II synthesis primer which anneals to its complementary sequence at the origin of replication and provides a free 3'OH group for DNA extension after RNase H cleavage (1). This process is down-regulated by RNA I, which hybridizes to RNA II, and the Rop protein further down-regulates DNA replication by stabilizing the hybridization between both RNA I and II (1). The G→A point mutation in the origin of replication decreases the ability for RNA I to hybridize to RNA II, leading to a higher copy number of pUC19 than pBR322. Additionally, the size difference between pBR322 and pUC19 has been suggested to have an impact on the difference in replication efficiency between the two plasmids since maintenance and replication of multiple plasmids leads to metabolic burden on host cells (3). Previous transformation studies suggest that transformation efficiency is inversely related to plasmid size as it has been shown that pUC19 has a higher transformation efficiency than pBR322 (4, 5). One explanation for this observation is that the larger pBR322 plasmid requires more energy and time to replicate than the pUC19 plasmid, consequently resulting with lower plasmid copy numbers over time (2). Alternatively, preferential maintenance of pUC19 may result due to the metabolic inefficiency for host cells to maintain different plasmids containing the same antibiotic resistance gene against ampicillin (1).

Previously, attempts to understand the effect of size on transformation efficiency have been made by creating deletion mutants of pBR322 to correspond closely in size to pUC19 (5, 6, 7). Other studies have attempted to explore the relevance of the *rop* gene by deleting or mutating the gene from pBR322, but have been inconclusive due to unsuccessful ligation and low DNA insert concentrations (8, 9,10, 11). Our study took a different approach as we attempted to construct pBART, an enlarged pUC19 vector, by inserting the *rop* gene and tetracycline resistance gene from pBR322 into the multiple cloning site of pUC19 (Figure 1).The insertion of the pBR322 fragment, containing the *rop* and tetracycline resistance genes, increased the size of pUC19 from 2686 bp to 4989 bp, which is comparable to the 4361 bp size of pBR322, while still maintaining the G→A point mutation in the original pUC19 origin of replication. Construction of the pBR322 fragment required manipulation of restriction sites due to findings in previous studies which showed that use of the NdeI restriction site in pBR322 does not result in successful ligation (5). Instead, we created a unique XbaI site in our pBR322 fragment for successful ligation into the pUC19 multiple cloning site. We hypothesize that plasmid size difference and presence of the *rop* gene decreases maintenance of pBR322 in DH5α cells and the construction of pBART will be effective in determining the influence of these factors on the selective maintenance of pUC19 over pBR322 when co-transformed. Correspondingly, future studies using pBART will enable better understanding of the influence of plasmid size and the presence of the *rop* gene on plasmid copy number differences between pUC19 and pBR322.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* DH5α cells were obtained from the Ramey strain collection of the Department of Microbiology and Immunology at the University of British Columbia and were grown in Luria-Bertani (LB) broth

or LB agar plates at 37ºC. When appropriate, 100µg/ml ampicillin (Sigma-Aldrich) and 12µg/ml tetracycline (Sigma-Aldrich) was also added to the growth media for selection of DH5α cells containing plasmids pUC19 and pBR322, respectively. For colony screening purposes, 2% w/v X-gal solution (Thermo Scientific) and 20% w/v IPTG (isopropyl β-D-thiogalactopyranoside; Thermo Scientific) was also added to growth media when appropriate.

Isolation of plasmids. Overnight cultures of *E. coli* DH5α-containing pUC19 and pBR322, provided by the culture collection of the Department of Microbiology and Immunology at the University of British Columbia, were grown at 37ºC in LB broth containing 100µg/ml ampicillin on a platform shaking at 180rpm. Isolation of plasmids pUC19 and pBR322 were performed using the Invitrogen PureLink® Quick Plasmid Miniprep Kit (Life Technologies) following the manufacturer's protocol. The plasmid DNA was eluted with sterile distilled water and concentration was quantified using a Nanodrop 2000c Spectrophotometer (Thermo Scientific).

Preparation of competent *E. coli* DH5α cells. For the preparation of competent *E. coli* DH5α cells, SOB media was prepared with 2% w/v tryptone, 0.5% w/v yeast extract, 8.56mM NaCl, 2.5mM KCl and 10mM $MgSO_4$ in distilled water and sterilized via autoclaving. CCMB80 transformation buffer was prepared with 10mM potassium acetate, 80mM $CaCl_2$, 20mM $MnCl_2$, 10mM $MgCl_2$, 10% v/v glycerol in distilled water, pH adjusted to 6.4 and filter sterilized via a 0.45µm pore size filter. *E. coli* DH5α cells were inoculated into 250ml SOB media and grown to an OD_{600} of 0.3 at 37ºC. The culture was then centrifuged down at 4ºC in order to pellet the cells. After decanting the supernatant, the cell pellet was then resuspended in 80ml CCMB80 transformation buffer, incubated on ice for 20 minutes and then centrifuged down to pellet the cells again. Finally, after decanting the supernatant, the pelleted cells were then resuspended in 10ml CCMB80 transformation buffer and aliquoted into 500µl vials before storage at -80ºC until heat shock transformation.

Primer preparation and PCR conditions. Primers were obtained from Integrated DNA Technologies®, Inc. and were designed to anneal to and amplify a 2321bp region of pBR322 containing both *tet* and *rop* genes. The forward primer sequence (5'-CATCGATAAGCTTTAATGCGG-3') included a HindIII restriction site found on the pBR322 plasmid. The reverse primer sequence (5'-AATCTAGAAGCGGAAGAGCGCCTG-3') included an XbaI restriction site that replaced a SapI restriction site on the pBR322 plasmid. PCR reactions were performed with reaction mixtures following the Invitrogen Platinum® *Pfx* DNA Polymerase (Life Technologies) protocol: 10X Invitrogen *Pfx* Amplification buffer, 10mM dNTP mixture, 50mM $MgSO_4$, 10µM forward primer, 10µM reverse primer, 100ng of pBR322 template, 1 U of Invitrogen Platinum® *Pfx* DNA Polymerase and sterile distilled water to a final volume of 50µl. Thermocycler conditions were as follows: initial denaturation at 94ºC for 3 minutes followed by 8 cycles of touchdown PCR with denaturation at 94ºC for 15 seconds, primer annealing at 61ºC to 54ºC at decreasing increments of 1ºC per cycle for 30 seconds, extension at 68ºC for 2 minutes 45 seconds, 27 more cycles with the annealing temperature at 53ºC, and then a final extension at 68ºC for 3 minutes. The pBR322 PCR products were purified using Invitrogen PureLink® PCR Purification Kit (Life Technologies) following the manufacturer's protocol. The PCR-purified products were finally eluted using sterile distilled water.

Restriction digest of pUC19 plasmid and pBR322 PCR products. Restriction endonucleases were used to create sticky ends for the pUC19 plasmid fragment within the multiple cloning site, as well as for the PCR amplified pBR322 plasmid fragment to allow for ligation of the two plasmid fragments. Restriction digest reactions were carried out using HindIII-HF®(New England Biolabs) and XbaI (New England Biolabs) in CutSmart™ buffer (New England Biolabs) at 37ºC for 2 hours. Restriction enzymes were then heat inactivated at 65ºC for 15 minutes.

Agarose gel electrophoresis of pUC19 and pBR322 fragments. The pUC19 and pBR322 fragments used to create the pBART construct were visualized on an agarose gel to confirm the identity of both fragments. The plasmid fragments were mixed with 6X DNA Loading Dye (Thermo Scientific) and loaded onto a 0.8% w/v agarose gel alongside a GeneRuler™ DNA Ladder Mix (Thermo Scientific) for band size comparison and was run for 50 minutes at 100V in 1X TAE buffer. The gel was stained in 0.5µg/ml ethidium bromide solution for 30 minutes before visualization and imaging with Alpha Imager (Multimage™ Light Cabinet).

Ligation of pUC19 and pBR322 fragments. Following the creation of sticky ends from the restriction digest of pUC19 and pBR322 fragments, the ligation of both fragments together was performed with Invitrogen T4 DNA Ligase (Life Technologies), following the manufacturer's protocol with the addition of a final concentration of 5% w/v polyethylene glycol (PEG) 6000 to the ligation reaction. Ligation reactions were carried out at room temperature for 1 hour before being heat inactivated at 65ºC for 15 minutes. The ligated products were then transformed via heat shock into competent *E. coli* DH5α cells and spread onto LB agar plates containing ampicillin, X-gal, and IPTG (LB-amp-Xgal-IPTG).

Restriction digest map of putative pBART plasmid constructs. Putative pBART plasmid constructs were subjected to a series of restriction enzyme digests in order to map out and confirm the identity of the plasmid construct. Isolated white colonies from the LB-amp-Xgal-IPTG plates were grown in overnight cultures of LB broth containing ampicillin and plasmids were isolated from each white colony using Invitrogen PureLink® Quick Plasmid Miniprep Kit (Life Technologies), following manufacturer's protocol. The plasmids were eluted with sterile distilled water. The putative pBART plasmids were then subjected to a series of restriction digests with the following combinations of restriction endonucleases in CutSmart™ buffer (New England Biolabs): HindIII-HF® (New England Biolabs), XbaI (New England Biolabs), MscI (New England Biolabs), HindIII-HF® & MscI, and XbaI & MscI. Control plasmids of wildtype pUC19 and pBR322 were also subjected to the same series of restriction digests in CutSmart™ buffer (New England Biolabs). The restriction digest reactions were all carried out at 37ºC for 2 hours, mixed with 6X DNA Loading Dye (Thermo Scientific), and then resolved on a 0.8% w/v agarose gel alongside a GeneRuler™ DNA Ladder Mix (Thermo Scientific) for 50 minutes at 100V in 1X TAE buffer. The gel was then stained in 0.5µg/ml ethidium bromide solution for 30 mintues prior to visualization and imaging with Alpha Imager (Multimage™ Light Cabinet).

Sequencing of *rop* gene in pBART plasmid construct. The region on the pBART plasmid construct expected to contain the *rop* gene was sequenced by GENEWIZ, Inc. The primer sequence designed for sequencing the *rop* gene (5'-CGCTATCGCTACGTGACTGG-3') was obtained from Integrated DNA Technologies, Inc. The sequencing reaction mixture was prepared by adding 20µM sequencing primer to 600ng of pBART plasmid template. Sequence alignment between the *rop* gene on pBART plasmid and the *rop* gene on pBR322 plasmid (NCBI accession no. J01749.1) was performed using the NCBI Nucleotide BLAST program suite.

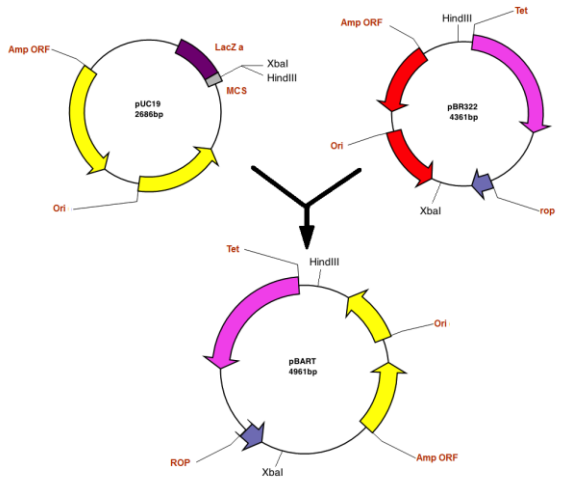

FIG 1 pUC19, pBR322 and the novel hybrid plasmid pBART. pUC19 fragment was generated by endonuclease digestion; pBR322 fragment was generated by PCR followed by endonuclease digestion. XbaI site is unique to pBART and does not exist in pBR322.

RESULTS

Amplification of 2.3 kilobase pair region of pBR322 containing the *rop* and *tet* genes using PCR. Annealing temperatures between 53°C and 61°C gave PCR products of the pBR322 plasmid at the predicted size. The expected size of the pBR322 plasmid fragment is estimated at 2.3 kb, which is slightly smaller than the predicted 2.6 kb pUC19 fragment. Gel electrophoresis confirms the prediction, as the pBR322 fragment generated by PCR migrated slightly further than the pUC19 fragment on the gel, indicating the pBR322 fragment is smaller than the pUC19 fragment (Fig. 2). In addition, the pUC19 band migrated above the 2.5 kb ladder band, consistent with the 2.6 kb predicted size. The pBR322 band migrated slower than the 2.0 kb ladder band, consistent with its predicted size of 2.3 kb.

Plasmid size of pBART confirmation by restriction enzyme digestion analysis. Both the pUC19 plasmid and the pBR322 fragment generated by PCR digested with restriction endonucleases and ligated to generate the novel hybrid plasmid pBART. Gel electrophoresis analysis showed a band slightly below the 5 kb ladder band, similar to the 4.9 kb predicted size of pBART. To further analyse pBART, double digests of the plasmid were performed followed by visualization of the digests from gel electrophoresis. A double digestion of HindIII and MscI gave two band sizes of 3.5 kb and 1.4 kb that correspond to the predicted sizes, with an additional 5 kb band from pBART due to incomplete digestion. A double digestion of XbaI and MscI gave two band sizes of 4 kb and 0.9 kb that correspond to the predicted sizes, with an additional 5 kb band due to incomplete digestion of the pBART plasmid. Digestion of pBR322 plasmid was also performed as a control on the specificity of the endonuclease activity. Single digests consistently showed a band size 4.3 kb, which is similar to the size of pBR322. A double digestion of HindIII and MscI gave two band sizes of 3 kb and 1.4 kb that correspond to the predicted sizes, with the third 4.3 kb band due to incomplete digestion of pBR322. Since pBR322 does not have an XbaI restriction site, double digestion using XbaI and MscI showed a 4.3 kb band that is similar to other bands with only a single digest (Fig. 3). Furthermore, *E.coli* DH5α strain transformed with pBART plated on LB-amp with X-gal and IPTG grew in white colonies, indicating *lacZ* disruption from the ligation of the insert. *E. coli* DH5α strain transformed with re-ligated pUC19 was plated as a negative control and growth of blue colonies was observed, indicating *lacZ* was intact due to the lack of pBR322 fragment insertion (data not shown). In addition, the sequencing result of pBART indicated the presence of *rop* gene, BLAST analysis showed the *rop* gene sequence is identical to that found in pBR322 (SF. 1). Together, the restriction map and gene sequencing allowed for confirmation of the construction of the novel hybrid, pBART.

Growth on LB-ampicillin plates but not LB-ampicillin-tetracycline plates indicates the lack of functional tetracycline resistance gene expression in pBART. The hybrid plasmid pBART is derived from pUC19 and pBR322 plasmids, which contain ampicillin and tetracycline resistance genes, respectively. However, *E. coli* DH5α strain containing pBART showed sensitivity to tetracycline as no growth was observed on the LB-ampicillin-tetracycline plate (data not shown); resistance to ampicillin was shown as growth was observed on the LB-ampicillin plate (data not shown). This indicates low or no tetracycline resistance gene expression in pBART to provide *E. coli* DH5α strain sufficient resistance to tetracycline.

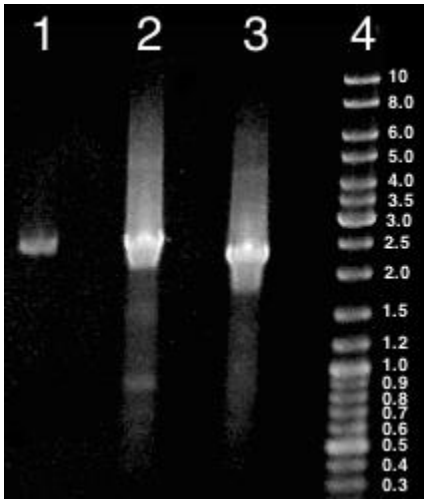

FIG 2 The band size of pUC19 fragment compared to pBR322 fragment generated by PCR. GeneRuler High Mass Ladder in lane 4 was used to determine the relative size of the bands. Lane 1 contains pUC19 fragment generated with double endonuclease digest. Lane 2 contains pUC19 fragment generated with PCR. Lane 3 contains pBR322 fragment generated with PCR.

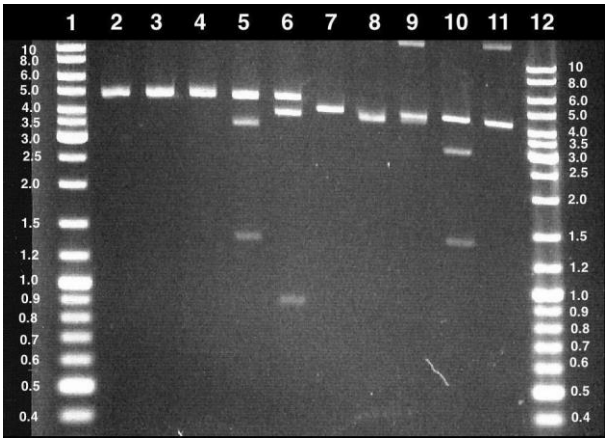

FIG 3. The band sizes of pBART construct compared to pBR322 plasmid under various endonuclease digestion conditions. GeneRuler High Mass Ladder in lanes 1 and 12 was used to determine the relative size of the bands. Lane 2 to 4 contain pBART single endonuclease digest (HindIII, XbaI, MscI, respectively). Lane 5 and 6 contain pBART with double endonuclease digest (HindIII/MscI and XbaI/MscI respectively). Lane 7 to 9 contain pBR322 single endonuclease digest (HindIII, XbaI, MscI, respectively). Lane 10 and 11 contain pBR322 double endonuclease digest (HindIII/MscI and XbaI/MscI respectively).

DISCUSSION

Construction of enlarged pUC19 vector. Previously, other groups have successfully managed to make constructs via manipulation of the pBR322 vector by mutating or deleting the *rop* gene in order to determine the functional effect of size and *rop* suppression on the plasmid copy number (7, 8, 9). In contrast to pBR322 manipulations, various groups have had difficulty enlarging the pUC19 vector and were ultimately unsuccessful (10, 11, 12, 13, 14, 15). The various authors have speculated that factors like low insert concentration and low ligation efficiency may have impeded attempts to create a working construct. Regardless, previewing the literature demonstrated a lack of usable pUC19 derived constructs to assist in determining the effect of size and *rop* expression on the plasmid copy number when compared to pBR322. As previously discussed, Lin Chao *et al*. have thus far been successful in showing a "gain of function" experiment, where the group manipulated pBR322 to demonstrate that deletion of the *rop* gene and mutation of G→ A in the origin of replication is necessary and sufficient to increase plasmid copy number of pBR322 to a level which is comparable to pUC19 (1). Based on their study, a similar prediction could be made by showing a "loss of function" experiment where the addition of the *rop* gene from pBR322 and mutation of A→G in the origin of replication of pUC19 would result in a decrease in plasmid copy number to a level that is comparable to pBR322. Therefore the successful construction of pBART, an enlarged pUC19 vector comparable in size to pBR322 with the presence of the *rop* gene, is the beginning of an experiment that can test whether a high plasmid copy number phenotype can be altered to a low plasmid copy number phenotype. Together, the restriction map and gene sequencing allowed for confirmation of the construction of the novel hybrid, pBART.

The importance of characterizing pBART. Currently little is known about the phenotypic properties of pBART itself. The results so far indicate that the pUC19 component of pBART is functional as the transformed DH5α cells grow in the presence of ampicillin and can replicate without any issues. However the results also indicate that the tetracycline resistance gene, *tet*, derived from pBR322 is non-functional (data not shown). A possible reason could be that the promoter sequence was lost during the cloning procedure to construct pBART. Without any promoter truncation experiments to investigate the location of the tetracycline promoter, it would be erroneous to rule out other possibilities including low level transcription from the IPTG-inducible *lac* promoter derived from pUC19 which runs in the opposite direction of the *tet* gene, which could potentially result in synthesis of anti-sense *tet* mRNA and silencing of the *tet* gene. Given the state of the non-functioning *tet* gene derived from the pBR322 component, the most prudent course of action would be verifying if the *rop* gene in pBART is functional. An interesting avenue to pursue in future studies would be determining and comparing the basal level of gene expression of *rop* for pBART to the *rop* gene in its parental wild type plasmid pBR322. The relative levels of the *rop* transcript expressed between the two plasmids could be quantified and would provide further insight on how similar or dissimilar pBART is from pBR322 in terms of expression levels of the *rop* gene. Furthermore, another parameter that has to be considered is the effect of plasmid size on transformation efficiency. Chan *et al.* have shown that increasing plasmid size results in decreasing transformation efficiency (4). The pBART vector is roughly 300 bp larger than pBR322 vector and it is expected that they should have comparable transformation efficiencies, however further experimentation is needed to test this prediction. Consequently, characterization of pBART is essential in understanding the basic properties of this hybrid construct as the knowledge can be used to set up the foundations for future experiments and ultimately assess its value in molecular cloning research.

FUTURE DIRECTIONS

pBART is a unique construct because it not only carries the critical G→ A point mutation from the origin of replication of pUC19, but it also constitutes of *rop* and *tet* genes from pBR322. Additionally unlike pBR322, pBART was designed to incorporate a unique XbaI restriction site

which was critical in distinguishing it from pBR322 (Fig. 3). This addition of a convenient XbaI unique restriction site will also allow greater flexibility in cloning for future experiments. The obvious step after the construction of pBART vector is to compare the plasmid copy number between pUC19, pBR322 and pBART. The result of such an experiment would be beneficial in validating the experiments carried out by Lin Chao *et al*. The authors had previously demonstrated in parallel that deletion of the negative regulatory *rop* gene from pBR322 and the G→A point mutation in the origin of replication independently played a role in increasing the plasmid copy number of the new construct compared to wild type pBR322 (10). Based on their results, it is tempting to speculate that pBART would have a lower plasmid copy number than pUC19 due to the addition of the *rop* gene which would decrease the replication levels of pBART, but would not be as low as pBR322 as the presence of the G→A point mutation would conversely increase the replication levels of pBART. Nonetheless, extensive data collection is required before such a conclusion can be made. Another line of experiments worth pursuing is reverting the G→A point mutation within the origin of replication of pBART to create a construct that is almost identical to pBR322. The plasmid copy number of the new construct could be compared with pBART itself and would be critical in determining the significance of the G→A mutation in increasing plasmid copy number. These experiments will be crucial in determining not only the elusive nature of the effect of the *rop* gene on the pUC19 vector but will also provide insight for other potential factors influencing plasmid copy number like plasmid size and the G→A point mutation within the origin of replication.

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