Journal of Experimental Microbiology and Immunology (JEMI) Vol. 18: 108 – 113

Introduction of a Specific Point Mutation into the RNA II Locus on a *rop*-deficient pBR322 Plasmid

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When the cloning vectors pUC19 and pBR322 are co-transformed into *E. coli* DH5α cells, pBR322 is excluded from the cells. This exclusion has been attributed to regulatory elements present in pBR322 that slow down plasmid replication compared to pUC19 where the same elements are absent or mutated. One of these elements is an RNA molecule called RNA I that hybridizes and sequesters the RNA II replication primer. Another of these elements is a protein called Rop that stabilizes the interactions between RNA I and RNA II. In pUC19, the Rop protein is not expressed and a point mutation in RNA II hinders its hybridization and sequestration by RNA I. A previous study focused on the role of Rop in the pBR322 exclusion effect by creating a *rop*-defient pBR322 derivative, but this construct did not show any mitigation of the exclusion effect. The current study created a *rop*-deficient pBR322 derivative that also contains the RNA II point mutation present on pUC19 so that the importance of this point mutation in the absence of Rop-stabilized RNA interactions can be investigated. The mutant plasmid called pGK13W was created using site-directed PCR mutagenesis, and a diagnostic restriction digest was performed to confirm the introduction of the desired point mutation.

Plasmids pUC19 and pBR322 are common cloning vectors derived from the plasmid pColE1 (1). When pUC19 and pBR322 are co-transformed into *E. coli* DH5α cells, however, pBR322 plasmids are excluded from the culture (1). This has been hypothetically attributed pUC19 having a faster replication rate and higher copy number (2). Differences in the replication of these two plasmids might be explained by the retention and modification of different elements of the replication machinery relative to their common source plasmid pColE1 (2).

In both pUC19 and pBR322, replication is primed by an RNA molecule transcribed from the RNA II locus. This molecule anneals to its complementary sequence at the origin of replication, providing a free 3' OH group for DNA extension. This process is down-regulated by a second RNA molecule that is transcribed from the RNA I locus. This second molecule is transcribed in 100-fold excess and is complementary to RNA II (2). Their hybridization results in the sequestration of primers molecules, thereby impeding replication. Interactions between the two RNA molecules are stabilized by the Rop protein to further down-regulate replication (1).

Two important differences in the pUC19 plasmid contribute to its faster replication rate. First, it carries a point mutation in its RNA II locus that changes the 112th base of the RNAII transcript from guanine to adenine (1). This mutation interferes with hybridization between the RNA II primer and the RNA I molecule. Second, the pUC19 plasmid does not express the Rop protein, so whatever interactions do occur between RNA I and RNA II are not stabilized (2).

Previous studies have focused on creating *rop*-deficient pBR322 plasmids and co-transforming these constructs with pUC19 and wildtype pBR322 to evaluate the importance of the Rop protein to the exclusion effect. In one study, a *rop*-deficient pBR322 plasmid called pIK054 was created by mutating the *rop* ribosome binding site using site-directed PCR mutagenesis (2). When pIK054 was co-transformed with pUC19 and wildtype pBR322, however, it was excluded by both pUC19 and pBR322 (3). This suggests that a *rop* deficiency alone might be insufficient to mitigate the exclusion effect, and that the RNA II point mutation might also have an important role.

Using pIK055 (a better-growing isolate from the same reaction as pIK054) as a template, the current study constructed a *rop*-deficient pBR322 derivative carrying the same RNA II point mutation as pUC19. A similar site-directed mutagenic PCR was used, and the resulting plasmid was named pGK13W. Site-directed PCR mutagenesis relies on carefully designed primers that are mostly complementary to the region where the mutation is desired, but carry purposefully mismatched bases that will replace existing bases in the template sequence. The result is the amplification of the whole plasmid into a linear PCR product that incorporates the mutations present in the oligonucleotide primers. The linear product is then transformed into a competent cell line where host enzymes ligate and circularize the plasmid (4). A diagnostic restriction digest was used to confirm the successful introduction of the desired point mutation into the RNA II locus in the current study.

MATERIALS AND METHODS

Isolation of plasmids. Overnight cultures of *E.coli* DH5α containing the plasmids pBR322, pIK051, pIK052, pIK053, pIK054, and pIK055 were grown in a shaking 37°C incubator for 16 hours in 3 ml of Luria-Bertani (LB) broth with 100 μg/ml ampicillin. Plasmids were isolated using the MP Bio RapidPURE™ Plasmid Mini Kit (cat# 112066400). All host strains were obtained from the MICB 421 culture collection in the Department of Microbiology and Immunology at the University of British Columbia.

Primer Design and PCR. The primers were designed to change the 112th base of the RNAII transcript from guanine to adenine. 18 complementary bases were provided on either side of the base mismatch so that the primers would remain annealed at the extension temperature, and the forward and reverse primer

were designed to anneal 'back-to-back' at the same location on their respective strands of the plasmid. The sequence of these primers and the region of the RNA II locus to which they anneal are presented in Figure 1. The Agilent Technologies – QuikChange Primer Design software was used to verify the melting temperature. The primers were synthesized by Integrated DNA Technologies, and the lyophilized primers were rehydrated to form 100 µM stock solutions. 100ng/µl solutions of both primers were prepared from the stock solutions.

The mutagenic PCR was performed using the conditions outlined in the QuikChange II-E Site-Directed Mutagenesis Kit (Cat# 200555; Revision D.01). One experimental reaction was prepared using 50ng of template, and the PCR was carried out using the cycle parameters outlined in Table 1. This was followed by a DpnI digested incubated at 37°C for 1 hour. A control DpnI digest was performed on 100 ng of template DNA. This control reaction and 20 µl of the PCR were visualized using agarose gel electrophoresis. A 1.5% w/v gel was prepared in 1X TAE, and it was run at 4V/cm in 1X TAE running buffer.

TABLE 1. Cycling parameters for the site-directed mutagenic PCR.

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	12	95°C	30 seconds
		55°C	1 minute
		68°C	4.2 minutes

Transformation. Following the DpnI digest, the PCR products were transformed into XL1-Blue Electroporation-Competent Cells as outlined in the QuikChange II-E Site-Directed Mutagenesis Kit (Cat# 200555; Revision D.01). The Bio-Rad Micro-Pulser was used for electroporation and was set at EC2 at 2.5 kV. The electroporation-cuvettes had a 0.2 cm gap. The experimental mutagenesis transformation reaction was plated on two LB + ampicillin (100µg/ml) plates. Two controls were set up with pWhitescript mutagenesis product and the kit-supplied pUC18 plasmid. The experimental and control plates were incubated overnight at 37°C. Two colonies were selected from each of the experimental plates. These were used to inoculate 3 ml cultures of LB with 100 µg/ml ampicillin that were incubated overnight at 37°C. Plasmids were isolated using the MP Bio RapidPURE™ Plasmid Mini Kit (cat# 112066400).

Restriction Endonuclease Digests and Agarose Gel Electrophoresis. Restriction digests were performed on pBR322, pIK055, and pGK13W DNA using AluI (cat# ER0011) from Thermo Scientific, Eco521 (cat# ER0331) from Thermo Scientific, and MboII (cat# FD0824) from Thermo Scientific. All digests were performed using 2 U of enzyme with its supplied buffer in a final volume of 20 µl. Approximately 350 ng of DNA were used in AluI and MboII digests, and these digests were incubated overnight at 37°C. The Eco521 digest was carried out on 100 ng of DNA and incubated for 30 minutes at 37°C. 20 µl of digest reactions were separated and visualized using agarose gel electrophoresis. 2% w/v gels were prepared in 1X TAE. These were run at 3V/cm in 1X TAE running buffer.

RESULTS

Host cells containing the pIK055 plasmid displayed the best growth on selective media. Five isolates of *E. coli* DH5α containing mutated pBR322 were selected from the original study that created Rop deficient plasmids (2). These plasmids were pIK051, pIK052, pIK053, pIK054, and pIK055. When each of these isolates was spread onto a selective plate, only 20 isolated colonies were observed on the plate containing pIK054 hosts. Between 100 and 200 colonies were observed on the plates containing pIK051, pIK052, and pIK053 hosts. Over 300 isolated colonies were observed on the plate containing pIK055 hosts. Due to this difference in growth on selective media, pIK055 was selected as the template for the current pGK13W construct.

A diagnostic restriction digest with AluI confirmed that the expected mutations were present in the pIK055 plasmid. An overnight culture of pIK055 host cells was grown and plasmids were extracted. A diagnostic restriction digest was performed to ensure that the plasmids contained the desired mutation in the *rop* ribosome binding site. These mutations were expected to introduce a recognition sequence for the AluI restriction enzyme into the pBR322 template. Plasmid DNA from pBR322 and pIK055 was completely digested with AluI, and the fragments were separated using agarose gel electrophoresis. These results are presented in Figure 2. They showed that the largest fragment from the digested pIK055 DNA was approximately 800 bp, while the largest fragment from the digested pBR322 DNA was approximately 900 bp and absent from the pIK055 digest. This is consistent with the expected results observed during the construction of pIK055 (2).

A site-directed mutagenic PCR product was successfully created and transformed into competent host cells. After confirming the presence of the *rop* mutations, a PCR was conducted to introduce the desired point mutation into the RNA II locus on pIK055 template DNA using the primers presented in Figure 1. Following thermocycling, the reaction was incubated with DpnI to digest the template DNA. Agarose gel electrophoresis was used to evaluate the effectiveness of the PCR. 20 µl of the product was run alongside 100 ng of template DNA partially digested with the restriction enzyme Eco521, showing both linear and supercoiled conformations of the template. A control DpnI digest of template DNA was run to verify that the *E. coli* DH5α host cells sufficiently methylated the template DNA for digestion by DpnI under the incubation conditions used. These results are presented in Figure 3. They showed a clearly defined band of DNA in the PCR product with the same mobility as the linear conformation of the 4361 bp template DNA. No bands were visible in the lane containing the control DpnI digest, indicating a full digest of 100 ng of template under the incubation conditions used.

A 2 µl aliquot of the PCR product was transformed into XL1-Blue electrocompetent *E. coli* cells via electroporation, and the transformation reaction was plated in duplicate on selective media. Nine isolated colonies were obtained on one plate, and six isolated colonies were obtained on the other. Two colonies were randomly selected from each plate and used to inoculate overnight cultures. Plasmids were then isolated from these cultures and named pGK13W-1, pGK13W-2, pGK13W-3, and pGK13W-4.

A diagnostic restriction digest with MboII confirmed that the mutagenic PCR inserted the desired point

FORWARD PRIMER: 3′ – GATGCCGATGTGATCTTCTTGTCATAAACCATAGACG – 5′

RNA II Locus: 5′ – CTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGC – 3′
3′ – GAACTTCACCACCGGATTGATGCCGATGTGATCTTCCTGTCATAAACCATAGACGCGAGACGACTTCG – 5′

REVERSE PRIMER: 5′ – CTACGGCTACACTAGAAGAACAGTATTTGGTATCTGC – 3′

FIG. 1. Forward and reverse primers for a site-directed mutagenic PCR of the RNA II locus on pIK055. These primers are designed to introduce a G to A point mutation at the highlighted bases.

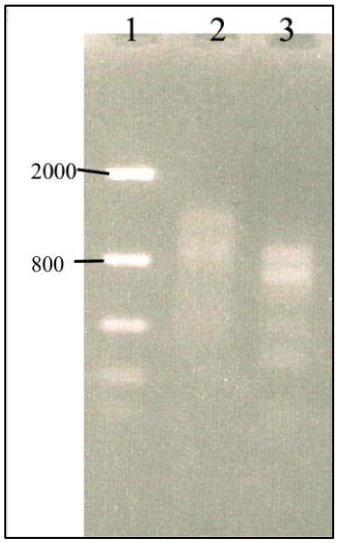

FIG. 2. Band pattern of pBR322 and pIK055 plasmid DNA digested with the restriction enzyme AluI. Lane 1 shows a low range linear DNA mass ladder, Lane 2 shows digested pBR322 DNA, and Lane 3 shows digested pIK055 DNA.

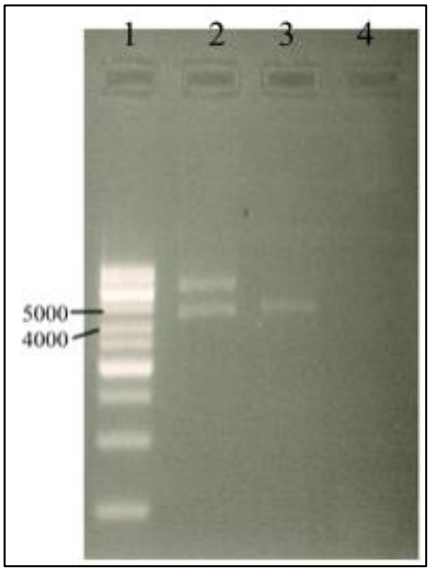

FIG. 3. **Band pattern of a site-directed mutagenic PCR using pIK055 plasmid DNA as a template.** Lane 1 shows a 1 kb linear DNA mass ladder, Lane 2 shows template DNA partially digested with Eco521 and present in both linear and supercoiled conformations, Lane 3 shows 20 µl of the mutagenic PCR product, and Lane 4 shows 100 ng of template DNA digested with the restriction endonuclease DpnI.

mutation. A diagnostic restriction digest was performed to confirm the successful introduction of the desired point mutation into the RNA II locus. This particular point mutation introduces a recognition site for the type IIs restriction endonuclease MboII. This restriction enzyme recognizes the sequence 5'-GAAGA-3' and cuts the DNA deoxyribose-phosphate backbone 8 nucleotides to the right on the top strand and 7 nucleotides to the right on the bottom strand (5). Under certain circumstances, however, cleavage is blocked by host methylation of the DNA. The MboII recognition sequence partially overlaps with the Dam methylase recognition sequence 5'-GATC-3', and the MboII protein covers a 19 nucleotide footprint on the DNA molecule (6). This means Dam methylation sequences at or near MboII sites will block cleavage of DNA.

Using this information, plasmid maps were constructed to show blocked and unblocked MboII restriction sites, along with the resulting fragments and their expected sizes. These maps are presented in Figure 4. They show that a 1617 bp fragment in pIK055 should be divided into 986 bp and 631 bp fragments by the creation of an MboII restriction site in the mutant pGK13W plasmid. The expected sizes of the fragments from both digested plasmids are tabulated in Table 2.

DNA from pIK055 and the four pGK13W transformants was completely digested with MboII and the fragments were separated using agarose gel electrophoresis. Two replicates of this digest are shown in Figure 5. The pIK055 lanes in Figure 5A and Figure 5B show a consistent banding pattern, with a ~1600 bp fragment present that is not visible in the pGK13W lanes. Similarly, a consistent banding pattern is visible in all but one of the pGK13W lanes, with ~950 bp and ~650 bp fragments that are not visible in the pIK055 lanes. With the exception of the pGK13W-1 lane in Figure 5A, all of the lanes in Figures 5A and 5B show banding patterns consistent with the expected fragment sizes outlined in Figure 4 and Table 2.

DISCUSSION

The comparatively poor growth of the pIK054 host cells offers a preliminary explanation of why past co-transformations with pIK054 did not produced the expected observations. As mentioned previously, when *rop*-deficient pIK054 was co-transformed with pUC19 and pBR322, it was unexpectedly excluded by both (3). In this study, pIK054 hosts displayed poor growth on selective media when compared to other plasmids from the same mutagenic PCR like pIK055 (a ten-fold difference). This raises the possibility that some feature

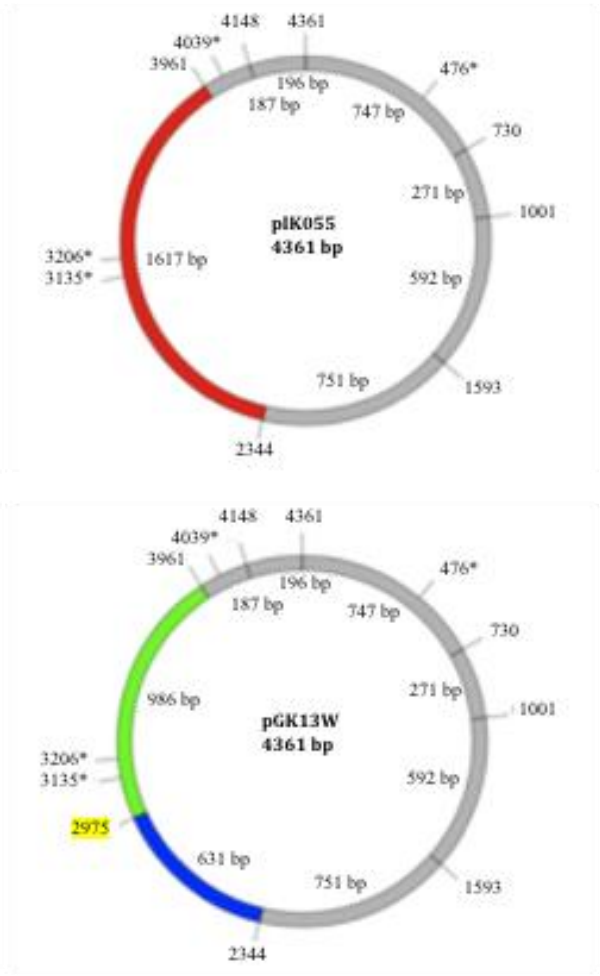

FIG. 4. **Plasmid maps of pIK054 and pGK13W showing MboII restriction sites and expected fragment sizes from a complete MboII restriction digest.** MboII sites blocked by host Dam methylation are asterisked, and the MboII recognition site generated by the mutagenic PCR is highlighted. The fragments affected by the introduction of this restriction site are coloured.

unique to the pIK054 plasmid or its host strain might cause it to replicate poorly, and that this unique feature might explain the unexpected exclusion of pIK054 by pUC19 and pBR322 in previous studies.

The results from the diagnostic restriction digest of pGK13W and pIK055 DNA with MboII show that the desired point mutation was inserted into the RNA II locus on pIK055. After separation of the fragments by agarose gel electrophoresis, all but one of the digests showed bands at the expected sizes. Figure 5 shows that a ~1600 bp band was present in the pIK055 lanes but absent from the pGK13W lanes. Conversely, ~950 bp and ~650 bp fragments are present in the pGK13W lanes but absent in the pIK055 lanes. This closely matches the changes in fragment size predicted in Figure 4 and Table 2. These results indicate that the desired point mutation was successfully introduced into RNA II, generating an additional MboII restriction site at the expected location.

One of the pGK13W-1 digests showed an unexpected result, as seen in Lane 3 of Figure 5A. This observation can be discounted as an outlier, likely due to an improperly prepared reaction resulting in an incomplete MboII digest. This conclusion is suggested by the presence of larger than expected bands and the absence of the smallest expected bands around 271, 196, and 187 bp. Futhermore, as can be seen in Figure 5B, a second replicate of the pGK13W-1 digest showed a trend consistent with the other pGK13W samples and the expected results outlined in Figure 4 and Table 2.

The results from this study also show that site-directed PCR mutagenesis can be an effective method for inserting point mutations into whole plasmids. A single PCR with 50 ng of template DNA generated sufficient product for visualization by agarose gel electrophoresis, as seen in Figure 3. The product migrated the same distance as the linearized template, confirming that it was in the expected linear conformation and of the expected 4361 kb size. Furthermore, the control DpnI digest in Figure 3 indicates that the lab strain of *E.coli* DH5α sufficient methylates DNA for digestion by DpnI, and it confirms that the PCR product contains exclusively mutant DNA. The control digest also indicates that the DpnI enzyme completely digested 100 ng of template in the control reaction under the incubation conditions used following the mutagenic PCR – twice the amount of template actually present in the mutagenic PCR. Indeed, when the PCR product was transformed into cells, plasmids in all of the four randomly selected colonies carried the desired point mutation.

FUTURE DIRECTIONS

The next step is to carry out the co-transformation experiment using pUC19, pBR322, pIK055, and pGK13W. The pGK13W plasmid created in this study has the desired guanine to adenine point mutation in RNA II that hinders hybridization with RNAI and it also lacks the *rop* gene. These two factors should synergize to impede RNA I from hybridizing with RNA II in pUC19. Since pGK13W has both these factors present, RNA II is unrestricted from acting as a primer and plasmid replication can happen more frequently. If the pBR322 exclusion effect is due to pUC19 having a higher copy number then pGK13W should have reduced exclusion during co-transformation with pUC19.

Additionally, the importance of a *rop*-deficiency alone should be further investigated using pIK055 instead of pIK054 since the significantly poor growth of pIK054 on selective media might indicate that it does not provide a representative model of a *rop*-deficient pBR322 plasmid. Co-transformation of pGK13W and pIK055 can also be

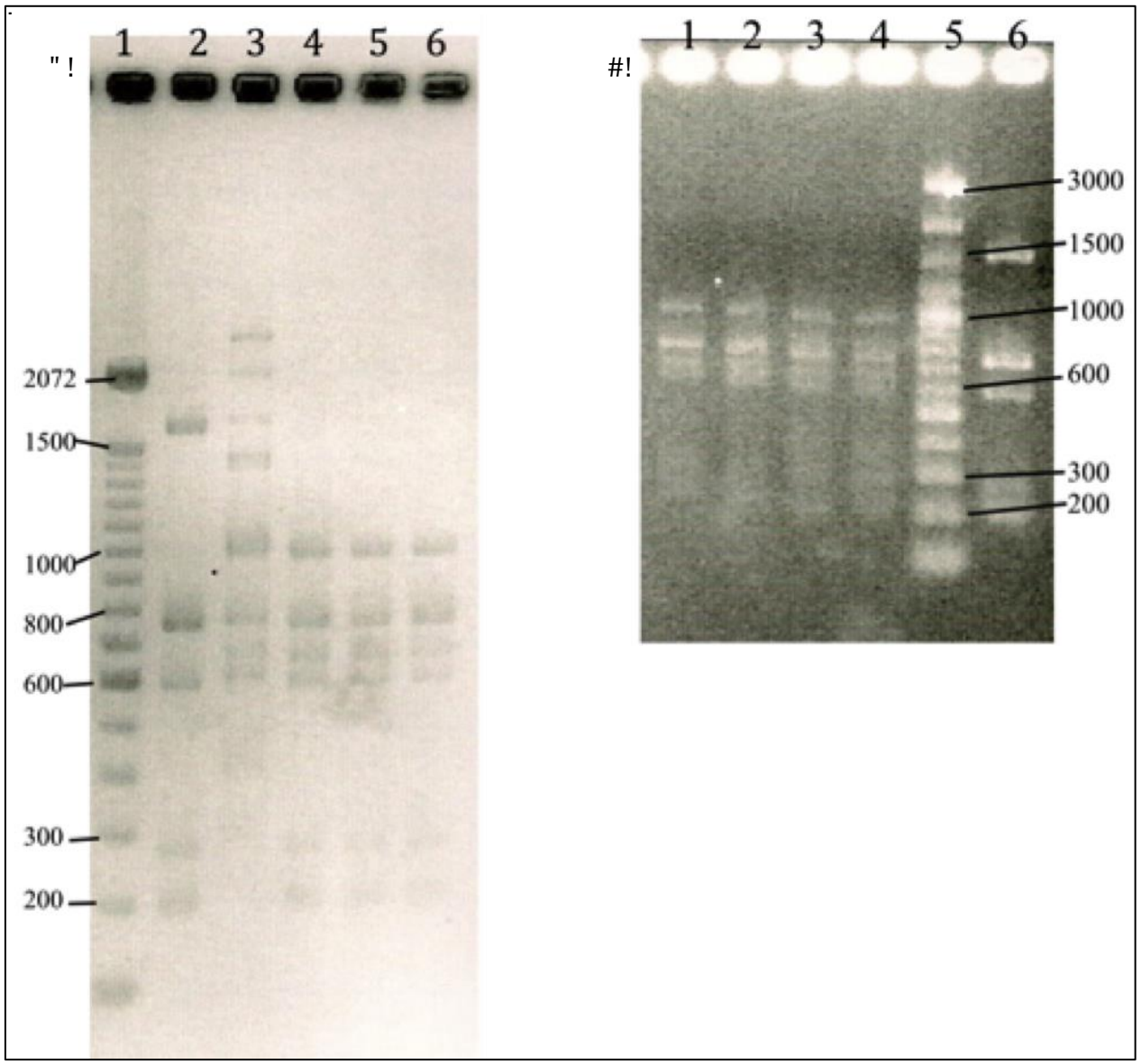

FIG. 5. **Band patterns of pIK055 and mutant pGK13W plasmid DNA completely digested with the restriction enzyme MboII and separated using agarose gel electrophoresis. A)** Lane 1 shows a 100 bp linear DNA mass ladder, Lane 2 shows digested pIK055 DNA, Lanes 3 – 6 show digested pGK13W-1, pGK13W-2, pGK13W-3, and pGK13W-4 DNA respectively. **B)** Lanes 1 – 4 show digested pGK13W-1, pGK13W-2, pGK13W-3, and pGK13W-4 DNA respectively, Lane 5 shows a 100 bp linear DNA mass ladder, and Lane 6 shows digested pIK055 DNA.

TABLE 2. Expected DNA fragment sizes from a complete digestion of plasmids pIK054 and pGK13W with the restriction endonuclease MboII. Fragments unique to a particular plasmid are asterisked. Fragments are listed in descending order by size in base pairs.

pIK055	pGK13W
1617*	986*
751	751
747	747
592	631*
271	592
196	271
187	196
	187

useful to evaluate the comparative importance of the point mutation and *rop*-deficiency.

These experiments can be carried out by co-transforming *E. coli* DH5α with pUC19, pBR322, pIK055, and pGK13W in varying amounts. The control co-transformation can be carried out by co-transforming *E. coli* DH5α with pUC19 and pBR322 for each variable amount of pGK13W and pIK055. This will allow for the direct comparison between pBR322 and pGK13W. If the exclusion effect is due to pUC19 higher copy number then pGK13W will show reduced exclusion during co-transformation and a higher colony count on the differential media selective for pGK13W.

Finally, it is not known to what extent the mutation in the *rop* binding site on pIK055 and pGK13W actually decreases the expression of *rop*. A western blot for Rop proteins from strains containing pGK13W, pIK055, and pBR322 would clarify this matter.

ACKNOWLEDGEMENTS

The authors would like to thank Dr William Ramey and Kirstin Brown for their invaluable insight and guidance, as well as lab mates who offered helpful tips and creative solutions to the various roadblocks along the way. This research was made possible with the support and funding of the Department of Microbiology and Immunology at the University of British Columbia.

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