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Construction of pCXZ14W, a Novel pUC19-derived Plasmid Encoding the *rop* Gene

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When the ColE1-derived cloning vectors pUC19 and pBR322 are co-transformed into *Escherichia coli* DH5α, pBR322 is excluded from the cells, meaning that overtime the plasmid copy number of pUC19 is higher than that of pBR322. This phenomenon has been attributed to the absence and modification of specific elements involved in the regulation of plasmid copy number, one of which is a protein called Repressor of Primer (Rop). Rop stabilizes the interaction between replication primer RNA II and its inhibitory RNA molecule, RNA I. In the absence of free RNA II plasmid copy number per cell decreases. To assess the role of Rop in the exclusion effect, several teams of researchers have attempted to construct a *rop*-deficient pBR322 plasmid. To date these efforts have been unsuccessful. The aim of this study was to create a novel plasmid, pCXZ14W, in which the *rop* gene is inserted into the pUC19 multi-cloning site (MCS). The construct can then be used to assess the effect of Rop on its copy number if Rop is expressed. Verified by both colony PCR and DNA sequencing, the *rop* gene was inserted into the pUC19 MCS without any mutation, though inadvertently being positioned at a distance of 52 nucleotides away from the Shine-Dalgarno sequence located in the *lac* operon. SDS-PAGE analysis showed no overexpression of Rop as expected. We next attempted to delete the sequence between the Rop start codon and the Shine-Dalgarno sequence to correctly position these elements for expression. 30 transformants were screened using colony PCR. However, clones bearing the deletion were not observed. We speculate that the deletion may have resulted in successful expression of the *rop* gene, driven from the upstream *lac* promoter, which downregulated plasmid copy number and rendered the desired transformant cells non-viable.

pUC19 and pBR322 are widely-used plasmid vectors in molecular cloning. When both plasmids are co-transformed into *Escherichia coli* DH5α, however, pBR322 is excluded and pUC19 is preferentially selected, meaning that the copy number of pUC19 outnumbers that of pBR322 (1). This phenomenon has been hypothetically attributed to pUC19 having a faster replication rate and therefore, higher copy number (2). The difference in the replication rate might result from the absence and modification of specific elements of the similar replication machinery shared by these two plasmids (2).

pUC19 and pBR322 are both derivatives of pColE1 plasmid, and shares a similar plasmid replication regulatory mechanism. Replication of ColE1-type plasmids starts with the synthesis of an RNA primer precursor called RNA II (1). In order to be an active primer, RNA II must bind to the template DNA, forming a DNA-RNA hybrid, followed by cleavage by the enzyme RNaseH to expose a free 3'OH end, which is accessible to DNA synthesis by DNA polymerase I (1). Another RNA molecule, RNA I, is transcribed as a 108 nucleotide sequence which is complementary to RNA II. RNA I can bind to RNA II, forming a RNA-RNA double helix to prevent RNA II from functioning as a primer (1). As a result, plasmid replication slows down and copy number is down regulated.

In addition to RNA I, a plasmid-encoded protein called Repressor of Primer (Rop) plays a role in regulating copy number. Rop stabilizes and enhances the pairing of RNA I and RNA II so that plasmid replication is inhibited even when the concentration of RNA I is relatively low (1). pBR322 carries the *rop* gene which encodes the Rop protein while pUC19 does not (1). It has also been shown that there is a point mutation in pUC19 RNA II sequence, which was originally thought to decrease the pairing efficiency of RNA II and RNA I, potentially leading to the elevated pUC19 copy number (1). However, it has been found that only in the absence of Rop does introduction of a corresponding point mutation into pBR322 result in elevated copy number, suggesting that the effect of this mutation can be suppressed by Rop (1).

The pBR322/pUC19 exclusion phenomenon might be due to the lack of *rop* gene in pUC19, which contributes to its elevated copy number, making pUC19 outnumber pBR322. Various approaches were attempted to create *rop*-deficient pBR322 and co-transforming mutant pBR322 and wild type pUC19 to investigate the effect of *rop* gene on the exclusion effect. However, to date these efforts have been unsuccessful (3).

The current study focused on building a rop^+ recombinant pUC19 plasmid to allow future investigation of the exclusion effect by co-transformation of rop^+ pUC19 and pBR322 plasmids into *E.coli* DH5α cells. Using pBR322 as the template, we constructed a recombinant pUC19 plasmid (pCXZ14W) with *rop* gene inserted into its multi-cloning site (MCS). A careful bioinformatics analysis revealed that the start codon of the *rop* gene and the Shine-Dalgarno (SD) sequence in the *lac* operon were inadvertently positioned at a distance of 52 nucleotides from each other. SDS-PAGE analysis confirmed that there was no overexpression of Rop in *E.coli* DH5α cells harboring pCXZ14W plasmid induced by IPTG. We next attempted to delete the sequence between the start codon of the *rop* gene and the SD sequence to decrease the spacing to six nucleotides using PCR-mediated plasmid deletion method. We screened all

yielded transformants using colony PCR but clones bearing the target deletion were not observed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E.coli* DH5α strain was used as the host strain for all cloning procedures. All strains were obtained through the MICB 447 Culture Collection from the Microbiology and Immunology Department at University of British Columbia. All strains were grown on Luria-Bertani (LB) agar plates (100 μg/ml ampicillin added for DH5α strains harboring plasmids) overnight at 37 °C.

Isolation of plasmids. *E.coli* DH5α strains harboring pBR322 or pUC19 plasmid were grown overnight in a shaking incubator in 5 ml of LB broth with 100 μg/ml ampicillin at 37 °C at 200 RPM. Plasmids were isolated using Invitrogen PureLink® Quick plasmid Miniprep Kit (Cat# K2100-10).

Preparation of *E.coli* DH5α competent cells. 1 ml of an overnight *E.coli* DH5α culture was used to inoculate 100 ml LB media until the OD_{600} reached a value of 0.4. The culture was chilled on ice for 10 min, followed by centrifugation for 3 min at 6000 rpm. The pellet was gently resuspended in 10 ml cold 0.1 M $CaCl_2$ and incubated on ice for 20 min, followed by centrifugation for 3 min at 6000 rpm. The pellet was gently resuspended in 5 ml cold 0.1 M CaCl2/15% glycerol and dispensed in microfuge tubes (100 μl/tube). The cells were stored at -80 °C.

Primer construction for *rop* amplification. pBR322 and pUC19 plasmid sequences was obtained from National Center for Biotechnology Information (NCBI) and *rop*-set primers were manually designed to amplify *rop* gene specifically using pBR322 as the template (Table 1, Fig. 1). XbaI and EcoRI restriction sites were added onto the 5' end of the forward and reverse primers, respectively. A polyhistidine-tag was fused in frame before stop codon so that Rop expression could be detected by Western Blot analysis using anti-polyhistidine antibody if SDS-PAGE fails to detect. The primers were synthesized by Integrated DNA Technologies, and the lyophilized primers were rehydrated to form 100 μM stock solutions. 10 μM solutions of all primers were prepared from the stock solutions.

TABLE 1 5' to 3' sequences of forward and reverse primers used to amplify specific gene or plasmid regions. Engineered restriction sites are underlined and are flanked at the 5' end by several bases for better restriction enzyme recognition and cleavage. Polyhistidine-tag is double underlined. F and R indicate forward and reverse primers, respectively.

Target/ Purpose	Primers (5' -> 3')
rop	F: GCGTCTAGAGTGACCAAACAGGAAAAA ACC
	R: TAACTAGAATTCTTAATGGTGATGGTGATGGT GGAGGTTTTCACCGTCATC
Deletion	F: GCGTCTAGAGTGACCAAACAGGAAAAAACC
	R: GCGTCTAGAAGCTGTTTCCTGTGTGAAATTG
Test	F: ATGACCATGATTACGCCAAGCTTGC
	R: TAACTAGAATTCTTAATGGTGATGGTGATGGT GGAGGTTTTCACCGTCATC

Polymerase chain reaction (PCR) amplification. The *rop* gene was amplified using the *rop*-set primers by PCR (Table 1). The reaction mixture of 50 μl contained 1X *Taq* Buffer with $(NH_4)_2SO_4$, 2.5mM $MgCl_2$, 0.2mM of each deoxynucleotide (dNTP), 1 μM of forward and reverse primers, 1.25 U *Tag* DNA Polymerase (Thermo Scientific), and approximately 50 ng of DNA template. PCR amplification was performed with a TGradient Thermocycler (Biometra) according to the following program: an initial denaturation at 95 °C for 3 min, followed by 30 cycles of 20 s at 95 °C, 30 s at 58 °C and 30 s at 68 °C; a final extension at 68 °C for 5 min. After PCR amplification, the PCR products were loaded into a 1% agarose gel (UltraPure agarose select agar, Invitrogen) containing 1X SYBR® safe DNA dye (Invitrogen) with 6X loading dye (Thermo Scientific) and ran for 45 min at 120 Volts. The agarose gels were visualized under the Alpha Innotech Corporation, MultiImage Light Cabnet. The Alpha Imager software was used to optimize and adjust the pictures for visualization.

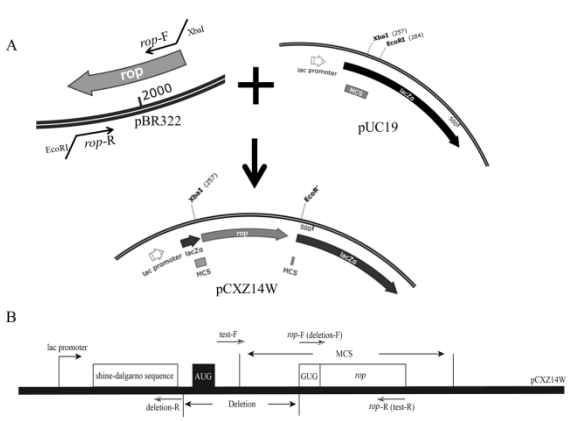

FIG 1 Cloning strategy. (a) The *rop* gene was first amplified using pBR322 as the template by PCR, followed by double digestion of pUC19 plasmid and amplified *rop* by XbaI and EcoRI restriction enzymes. Then digested vector and insert was ligated by T4 DNA ligase and the ligation product was transformed into *E.coli* DH5α cells for blue/white selection. Only the relevant regions of plasmids are shown. (b) A close-up view of lac operon of pCXZ14W plasmid with *rop* gene fused in. AUG in the black box represents the start codon of β-galactosidase gene and GUG in the white box represents the start codon of cloned *rop* gene. The locations of all primer sets are marked with F and R representing forward and reverse, respectively. MCS indicates multi-cloning site of pCXZ14W plasmid.

Purification of PCR amplified fragments, digestion and ligation. PCR amplified fragments, confirmed by gel electrophoresis, were purified using Invitrogen PureLink® PCR Purification Kit (Cat# K3100-01). Concentrations and purity of purified products were determined by Nanodrop 2000c spectrophotometer (Thermo Scientific). Amplified *rop* gene and pUC19 plasmid were double digested by the restriction enzymes EcoRI and XbaI (New England Biolabs). The 20 μl digestion mixture contained 1 μl of each restriction enzyme, approximately 500 ng of DNA, and 1X CutSmart Buffer (New England Biolabs). Both digestions were carried out for 60 min at 37 °C, followed by heat inactivation at 80 °C for 20 min and purification using Invitrogen PureLink® PCR Purification Kit before ligation. Double digested pUC19 plasmid and *rop* were ligated using T4 DNA ligase with a 3:1 molar ratio of insert and vector. The ligation reaction mixture contained 1 U of T4 DNA ligase (Invitrogen), 1X T4 DNA ligase buffer (Invitrogen) as well as 40 ng of pUC19 and 9 ng of *rop*. The ligation reaction was incubated overnight (18 hours) at 16 °C.

Transformation of *E.coli* DH5α competent cells. 2 μl of the ligation product was added into 100 μl *E.coli* DH5α competent cells. The cells were incubated for 30 min on ice, followed by heat shock for 1 min at 42 °C and incubation on ice for another 2 min. After that, 400 μl of LB medium was added and the cells were recovered at 37 °C for 1 hour on a shaking incubator, followed by centrifugation for 3 min at maximum speed. 250 μl supernatant was taken out. The cells were resuspended in the remaining media and plated on LB agar plate with 100 μg/ml ampicillin. 100 μl of 1000X X-gal stock solution and 10 μl of 0.1

M IPTG stock solution were also plated if blue-white screen ing looking for the clones containing recombinant DNA would be carried out.

DNA sequencing of construct. After blue-white screening, several white colonies were picked and inoculated in 5 ml of LB broth with ampicillin. The cultures were grown overnight at 37 °C, followed by plasmid isolation. 10 µl of 50 ng/µl plasmid was loaded into PCR strip tubes and sent for sequencing at GeneWiz. The universal primers M13F (-21) and M13R from GeneWiz were used to prime the sequencing reaction.

IPTG induction. 1 ml of an overnight *E.coli* DH5α strain either harboring recombinant plasmid or wild type pUC19 plasmid was inoculated into 99 ml of LB broth with ampicillin, respectively, until the OD_{600} reached a value of 0.6. Both cultures were then split into four portions with equal volume. IPTG was added into three cultures in each group to reach a final concentration of 0.5 mM, 1 mM, and 1.5 mM, respectively, followed by incubation for 2 hours at 37 °C. 1 mL of each culture was pelleted and stored at -20 °C for SDS-PAGE analysis.

SDS-PAGE analysis. Since the Rop protein with polyhistidine-tag fused at carbonyl-terminus has a relatively small size of approximately 8 kDa, a modified 18% SDS-PAGE protocol adapted from Thermo Scientific (4) was used to improve the resolution of small proteins (Table S1). All pellets obtained from IPTG induction were boiled at 95 °C for 15 min in 4X sample buffer (0.125 M Tris pH 6.8, 5% v/v beta-mercaptoethanol, 2% w/v SDS, 0.02% w/v bromophenol blue, 10% w/v glycerol). The lysates (approximately 20 µg protein per lane) were loaded onto 18% SDS gels along with protein molecular weight standards. The gels were run at 50 V for 30 min, followed by 2 hours at 150 V in running buffer (25 mM Tris base, 190 mM glycine, 1% w/v SDS at pH 8.3).

Deletion of intervening sequence. A specific region located in *lac* operon was targeted for deletion to reposition the start codon of *rop* sequence and the Shine-Dalgarno (SD) sequence. Therefore, a second set of primers, deletion-set, was designed (Table 1). The forward primer was the same as the one used in the *rop*-set. The reverse primer was designed as the reverse complement of a sequence corresponding to 22 bases upstream of the plasmid DNA to be deleted followed by *Xba*I restriction site (Fig. 1). Primers were added to a 50 µl PCR reaction mixture containing the same components as in *rop* amplification except that 50 ng of recombinant pUC19 plasmid instead of wild type pUC19 was added. PCR amplification was performed using the same program as in *rop* amplification except that the extension step requires 3 min to complete for each cycle. After amplification, PCR products were visualized and confirmed on a 1% gel, followed by purification using Invitrogen PureLink® PCR Purification Kit. 300 ng of purified fragments were then single digested by *Xba*I restriction enzyme, followed by self-ligation using T4 DNA ligase. Afterwards, 2 µl of ligated plasmids were transformed into 100 µl *E.coli* DH5α competent cells.

Colony PCR confirmation of recombinant pUC19 plasmid with target region deleted. Colony PCR as performed in order to screen colonies harboring recombinant plasmids with target region deleted. The third set of primers, test-set, was designed (Table 1). The reverse primer was the same as the one used in the *rop*-set whereas the forward primer anneals to the region targeted for deletion (Fig. 1). PCR amplification was carried out using the identical reaction mixture and cycle program as in deletion process except that one colony, instead of template DNA, was picked and directly added into the reaction mixture.

RESULTS

Construction of pUC19-derived plasmid encoding the *rop* gene. Since both melting temperatures of forward and reverse primers in the *rop*-set were between 62 °C and 65 °C, an annealing temperature of 58 °C was chosen in the PCR cycle program. The only PCR product amplified using pBR322 as the template had a size of slightly over 200 bp, which matched the size of *rop* with polyhistidine-tag fused, 230 bp (Fig. 2).

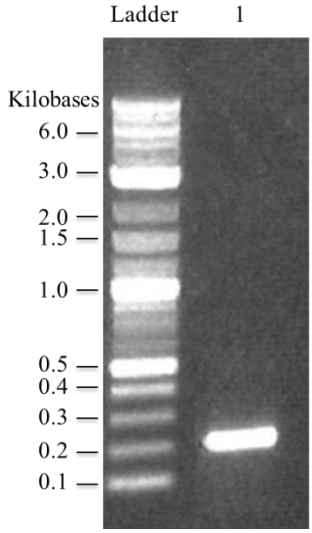

FIG 2 1% agarose gel showing PCR amplification of ~ 200 base pair band predicted to contain the *rop* sequence. Lane 1 contains the PCR product. 2-Log DNA Ladder (NEB) was used.

Amplification of *rop* was successful in yielding high amounts of DNA. However, PCR purification using Invitrogen PureLink® PCR Purification kit consistently resulted in considerable reductions in concentrations prior to double digestion by XbaI and EcoRI (data not shown). Therefore, multiple PCR reactions were pooled during the PCR purification process, leading to an increase in DNA concentration after the purification step.

pUC19 plasmid was also double digested by XbaI and EcoRI restriction enzymes, followed by the PCR purification in order to prevent small fragments re-ligating back to the plasmid during the ligation step. Relatively high concentration of DNA was yielded after the purification (data not shown). Purified double digested pUC19 and the *rop* amplicons were ligated by T4 DNA ligase using a vector-to-insert molar ratio of 1:3, followed by transformation into *E.coli* DH5α cells. To screen for clones lacking the β-galactosidase activity, which indicates the insertion of foreign DNA, X-gal and IPTG were also added to the agar plate. Approximately 30% of all yielded colonies were blue and the rest were white (data not shown).

DNA sequencing of plasmids obtained from several white colonies was carried out by GeneWiz using both forward and reverse universal primers. The correct sequence of *rop* with polyhistidine-tag fused in frame was aligned to the plasmid sequences obtained from GeneWiz

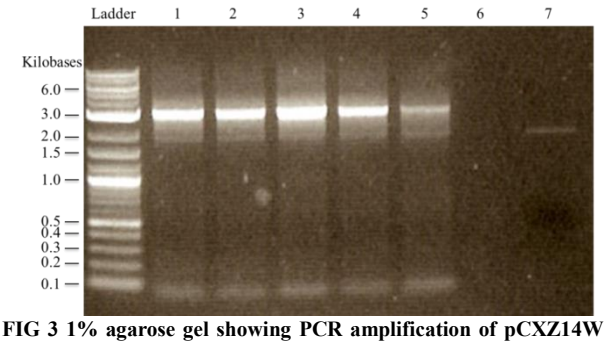

FIG 3 1% agarose gel showing PCR amplification of pCXZ14W plasmid fragment with intervening sequence deleted. Lanes 1-5 contain PCR products using pCXZ14W as the template. Lane 7 contains the pCXZ14W template diluted to the same extent as in PCR reactions. 2-Log DNA Ladder (NEB) was used.

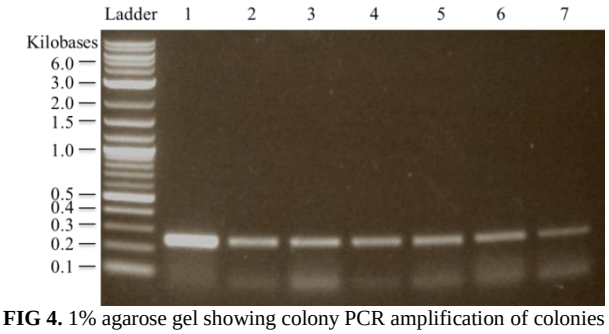

FIG 4. 1% agarose gel showing colony PCR amplification of colonies harboring pCXZ14W plasmid with intervening sequence potentially deleted. Lane 1 (control) contains PCR products using wild type pCXZ14W plasmid as the template. Lane 2 (control) contains colony PCR products using one single colony harboring wild type pCX14W as a source of template. Lane 3-7 contain colony PCR products using transformed colonies as a source of template. 2-Log DNA Ladder

using BLAST tool (NCBI). The forward sequencing yielded 98% identity (Fig. S1). The trace file confirmed that 2% difference was not due to any mutation in our insert but a result of low sequencing quality at the end of sequence. The reverse sequencing yielded 100% identity (Fig. S1), which confirmed proper *rop* insertion and the fidelity of PCR.

Taken together, all these data suggested that the *rop* gene amplified using pBR322 as the template was inserted into pUC19 plasmid. The *rop-6xHis*$^+$ recombinant pUC19 plasmid was denoted as pCXZ14W (Fig. S2). We performed a careful bioinformatics analysis on the pCXZ14W plasmid sequence obtained from GeneWiz and found that the start codon of the *rop* gene and the SD sequence were inadvertently positioned a distance of 52 nucleotides from each other which exceeds the optimal five nucleotides spacing for expression (5).

Attempted deletion of the intervening sequence between the start codon of the *rop* gene and the Shine-Dalgarno sequence. We sought to delete the intervening sequence between the start codon of the *rop* gene and the SD sequence to decrease the spacing of two elements to six nucleotides. We carried out PCR-mediated plasmid deletion method (Fig. S3). The reverse primer was designed as the reverse complement of a sequence upstream of the plasmid DNA to be deleted. The forward primer was the same as the one used in amplifying *rop*, which anneals to 5' of *rop* sequence downstream of the plasmid DNA to be deleted (Table 1). The plasmid fragment with the intervening sequence deleted was amplified with non-trivial amount of non-specific products (Fig. 3). As shown in Fig. 3, the major PCR product had a size of slightly below 3000 bp, which matched the size of plasmid fragment with the intervening sequence deleted. Compared with the original pCXZ14W template, all PCR products had small amount of templates left. However, since amplified plasmid fragment was far more abundant than the template, we did not gel extract the amplicons. We digested amplified pCXZ14W plasmid fragment using XbaI restriction enzyme, followed by the PCR purification, self-ligation using T4 DNA ligase and transformation into *E.coli* DH5α cells. All reactions were performed using the same conditions as in pCXZ14W construct assembly and yielded sufficient products (data not shown). 30 colonies were observed on the transformation plate.

Due to time constraints, colony PCR was carried out to screen colonies in a more efficient way. We designed forward primer to specifically anneal to the plasmid DNA to be deleted (Table 1). The same reverse primer was used as in *rop* amplification, which anneals to 3' end of *rop* sequence (Fig. 1). Therefore, colonies harboring pCXZ14W plasmid with intervening sequence deleted would not show any amplification whereas those harboring wild type pCXZ14W plasmid would. We screened 30 colonies and all of them showed amplifications, indicating that they did not bear deletion (Fig. 4). As can be seen from Fig. 4, both controls (lane 1 and 2) yielded PCR amplified fragments with a size of slightly over 200 bp, which was as expected because the intervening sequence was not deleted. However, all transformed colonies (only five shown) also showed amplification with the same size, suggesting that the plasmid DNA to be deleted was still present.

SDS-PAGE analysis showed no overexpression of Rop protein in IPTG-induced *E.coli* DH5α cells harboring pCXZ14W plasmid. We sought to confirm the expression status of Rop by performing SDS-PAGE analysis. Both *E.coli* DH5α cells harboring either pUC19 or pCXZ14W plasmid were induced by different concentrations of IPTG. Lysates of each culture were loaded into a SDS-PAGE gel. As demonstrated in Fig. 5, there was no difference observed between induced and non-induced *E.coli* DH5α cells harboring pCXZ14W plasmid and no overexpression of Rop protein (8kDa) could be observed. Western Blot analysis using anti-polyhistidine tag antibody was not carried out to further verify the expression status of Rop due to time constraints.

DISCUSSION

We attempted to construct a *rop*$^+$ pUC19 plasmid which can express Rop protein after transformed into *E.coli* DH5α cells. If Rop protein expression can be induced

by IPTG, co-transformation of *rop*$^+$ pUC19 and pBR322 plasmid can be carried out to assess the role of Rop protein in the exclusion effect. We amplified *rop* gene using pBR322 as the template. After screening for clones lacking the β-galactosidase activity, DNA sequencing confirmed the insertion of *rop* with polyhistidine-tag in frame into pUC19 MCS. However, the start codon of the *rop* gene and SD sequence were 52 nucleotides away from each other, potentially making *rop* unrecognizable by the host translation machinery.

SDS-PAGE analysis confirmed no overexpression of Rop protein in *E.coli* DH5α cells harboring pCXZ14W plasmid (Fig. 5). Therefore, we sought to delete the intervening sequence from the start codon of β-galactosidase (Fig. 1) to the base before the start codon of *rop* to bring *rop* in close proximity to SD sequence to be recognized. After deletion, the spacing of SD sequence and the start codon of *rop* would be six nucleotides, which is close to the optimal distance, 5 nucleotides (5).

We used PCR-mediated plasmid DNA deletion (Fig. S3). pCXZ14W plasmid fragment with intervening sequence deleted was amplified, as confirmed by gel electrophoresis (Fig. 3). However, a non-trivial amount of pCXZ14W template was still present in PCR products. Since PCR amplicons were much more abundant, gel purification was not carried out. After transformation, due to time constraints, screening was performed using colony PCR which used a negative result (i.e. no amplification) as an evidence for target deletion. In future experiments, a stronger screening method such as DNA sequencing should be carried out to obtain more reliable results. We screened all 30 colonies, none of which showed target deletion.

We suggest two possible explanations for this result. First of all, the template left in the PCR products was not eliminated, which could be carried into subsequent steps. XbaI restriction digest could linearize the template, but the following ligation step could re-ligate the template, leading to the transformation of the original template. Thus, colonies bearing no target deletion could grow. However, as mentioned before, amplified pCXZ14W fragment was far more abundant than the leftover template. As a result, even though there could be colonies harboring the original pCXZ14W, a larger portion of colonies should bear deletion. In contrast, all colonies we screened did not bear deletion. The second possible explanation is that the expression of Rop protein is lethal to cells. Rop, as mentioned before, downregulates plasmid copy number. If some pCXZ14W plasmids with intervening sequence deleted were transformed into cells and the deletion successfully made Rop recognized and expressed, theoretically Rop would start downregulating

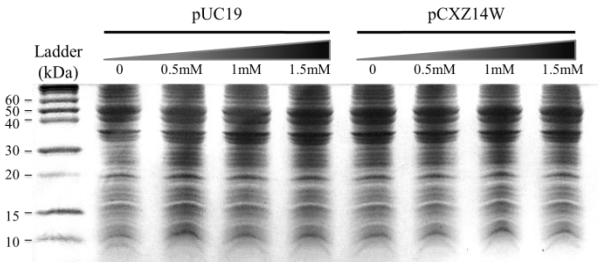

FIG 5 18% SDS-PAGE gel showing protein expression profiles of *E.coli* DH5α cells harboring either pUC19 or pCXZ14W plasmid induced by IPTG or not. 1 ml culture of each group was pelleted and boiled in SDS sample buffer. Approximately 20 μg protein from each group was loaded into SDS-PAGE gel. Novex® Sharp Pre-stained Protein Standard was used. (NEB) was used.

pCXZ14W plasmid. Therefore, the downregulation could decrease the copy number of ampicillin resistance gene carried in the plasmid, eventually causing cells to die due to the lack of antibiotic resistance.

However, IPTG was not added onto the transformation plate. Even though deletion was successful, Rop could not be expressed due to the lack of inducer. We next sought to see how leaky the *lac* operon is without IPTG induction. It is striking that several scientific papers have pointed out that the strength of control over gene expression from *lac* promoter is low which can lead to basal expression of downstream gene (6, 7). Therefore, perhaps the level of basal transcription was high enough to allow Rop to be expressed, which in turn downregulates pCXZ14W plasmid copy number to the extent that cells could not survive in the presence of ampicillin.

It is also worth noting that the start codon of *rop* sequence is GTG instead of the prevalent classic ATG codon. Approximately 14% of start codons used in *E.coli* is GTG (8). There is a lack of research assessing the reason why GTG start codon is preferentially used instead of ATG in some specific situations. However, one research group compared the translation efficiency of adenylate cyclase using UUG, GUG or AUG as its start codon, obtaining a translation ratio of 1:2:6, which suggested that AUG is the most efficient one (9). In addition, the fact that GTG has a lower translation efficiency has been demonstrated in mammalian cells as well (10). Therefore, it is tempting to speculate that a different start codon is used when the gene should be tightly regulated. In other words, bacteria decrease the expression of certain genes which can cause adverse effects when expressed at a higher level using alternative start codons that can be recognized by translation machinery less efficiently. It is possible that same regulatory control is involved in regulating *rop* gene expression.

In conclusion, we have constructed a *rop*$^+$ pUC19 plasmid, with the *rop* gene located 52 nucleotides downstream of the SD sequence in the *lac* operon. We

have attempted to delete the intervening sequence that prevents *rop* from being recognized by the host translation machinery. Although the target sequence has not been deleted, our construct may serve as a control plasmid which can be used in subsequent experiments investigating the lethality of Rop overexpression.

FUTURE DIRECTIONS

Our results raised the possibility that Rop protein expression is lethal to *E.coli* cells and the *lac* operon has leaky basal transcription, which potentially explains the reason why all transformants did no bear the target deletion. However, it is also possible that it was our deletion strategy that resulted in false positive colonies. Therefore, it is essential to eliminate any pCXZ14W template left in the PCR products. There are various approaches that can be attempted. For example, since the plasmid DNA to be deleted contains a portion of MCS, restriction enzymes that only recognize sites within this portion could be used to linearize the original template without affecting circularized amplified pCXZ14W plasmid fragment. Also, since *E.coli* DH5α strain is methylation positive, DpnI restriction enzyme can also be used to eliminate methylated template without affecting non-methylated amplified fragments. Once template has been removed with confidence, if the transformation result still yields false positive colonies or no colony, Rop protein being lethal can be supported. To further verify the lethality of Rop, *rop* gene can be inserted into a tightly-regulated expression vector. For example, *rop* gene can be inserted into the pET expression system under the control of T7 promoter, followed by transformation into *E.coli* DH5α cells. Since DH5α strain is lack of T7 polymerase (12), T7 promoter cannot be recognized, thereby eliminating the basal transcription of *rop*. Then the recombinant pET vector can be transformed into T7 polymerase-containing strain such as BL21 (DE3) to allow Rop to be expressed upon induction (12). The transformation can then be compared to assess the lethality of Rop protein. However, one essential point that must be taken into account is that Rop protein may not regulate plasmid copy number if the plasmid is not under the control of the same regulatory mechanisms. Since pET vector is pBR322-derived (13), same plasmid copy number regulatory control is likely to be employed.

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