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Attempted construction of a *soxR::*P_{soxS}*::lacZ* recombinant pBR322 plasmid as a sensitive reporter of internal reactive oxygen species in *Escherichia coli* DH5α

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This study attempted to engineer an internal reactive oxygen species probe using *lacZ* expression driven by the *soxS* promoter in response to intracellular superoxide presence being sensed by SoxR. Using polymerase chain reaction amplification, *soxR* fused to the promoter of *soxS* (P_{soxS}) and *lacZ* amplicons were obtained. Double restriction digests and sticky-end ligations were then performed in an attempt to clone *soxR::*P_{soxS}*::lacZ* into the pBR322 plasmid vector. Following ligation, transformation of competent *Escherichia coli* DH5α by calcium chloride-heatshock transformation was performed, and resulting transformants were screened for the *soxR*::P_{soxS}*::lacZ::pBR322* construct. Even though the amplicons were made, a successful ligation was not achieved. The lack of success in obtaining the desired construct prevented further examination of the potential of the proposed reactive oxygen species probe.

Reactive oxygen species (ROS), such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), are radicals that can be generated by various mechanisms including exposure to UV-A, paraquat, and side reactions involving quinones from the electron transport chain (1,2,3). Excessive levels of ROS are potentially harmful towards microorganisms due to the indiscriminate reactivity of ROS with many cellular processes within the cell including protein oxidation (1,3,4). As such, many bacteria have developed mechanisms in order to prevent the accumulation of ROS within the cell, particularly the OxyR and Sox regulons.

The OxyR regulon is involved in the sensing and metabolism of H_2O_2 within *Escherichia coli*. The OxyR regulon uses OxyR as the ROS sensor to detect intracellular levels of H_2O_2 (5,6). Upon detection of H_2O_2, OxyR induces the expression of both *kat*G and *kat*E which encode HPI and HPII, respectively (7). HPI and HPII are catalases that catalyze the conversion of H_2O_2 into water and molecular oxygen (8). In previous studies, the limited sensitivity of a *kat*G promoter based probe led to the investigation of alternative candidates of ROS sensors, which incurred the investigation of using SoxR as the sensor for internal ROS (9).

The Sox regulon uses the SoxR homodimer as the ROS sensor to detect intracellular levels of O_2^- (10). Upon detection of O_2^-, two [2Fe–2S] are oxidized activating SoxR homodimer (7). In both the reduced and oxidized states, SoxR binds upstream of the *soxS* promoter, but only the oxidized state is able to increase the expression of SoxS which in turn upregulates the expression of several genes involved in O_2^- metabolism including a manganese superoxide dismutase (11). Previous studies have shown that *soxS* upregulation in the presence of O_2^- is greater than the upregulation of *kat*G in the presence of H_2O_2 (12). Because of the higher level of constitutive expression and induction of the Sox regulon in response to O_2^- in comparison to the OxyR regulon, we attempted to construct a plasmid with a SoxR sensor, and *soxS* promoter driven LacZ expression as a more sensitive ROS probe compared to the previously studied *kat*G*::luxCDABE* probe (9).

A possible reason for the lack of sensitivity of the *kat*G*::luxCDABE* probe used in a previous study may be a consequence of luminescence activity inhibited by the growth media (9). We decided to create a new probe by using *lacZ* instead of *luxCDABE*,, in pursuit of generating an even more sensitive ROS probe. *lacZ* is a gene for β-galactosidase (LacZ), an enzyme that hydrolyzes β-galactosides into monosaccharides. When 4-methylumbeliferryl-β-D-galactopyranoside (MUG), a chromogenic substrate, is cleaved into 4-methylumbelliferone (MUB) and galactose, a chromophore is produced that excites at 390 nm producing fluorescent emission at 460 nm (13). Because the β-galactosidase assay used in conjunction with MUG has been shown to have a detection limit of 2 pg of β-galactosidase, this new probe should be more sensitive than the *luxCDABE* gene cluster which has been shown to have its luminescent activity reduced by growth media(14).

The purpose of this study was to use polymerase chain reaction (PCR) to amplify *soxR* fused to the *soxS* promoter (abbreviated in this report as *soxR::*P_{soxS}) and *lacZ* from *E.* coli MG1655 genome in order to construct a recombinant *soxR::*P_{soxS}*::lacZ* probe. This recombinant probe was to be used in future studies to determine the sensitivity and viability of its use as an internal ROS probe that would be expressed from the recombinant pBR322 plasmid vector once transformed into *E. coli* DH5α.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strain MG1655 was used to amplify genes of interest for constructing our plasmid, and the *E. coli* strain DH5α was used to transform our construct, as it lacks an active β-galactosidase. Both strains were obtained from the MICB 421 Culture Collection in the Microbiology and Immunology Department at University of British Columbia, then streaked onto a Luria-Bertani (LB) agar plate and were grown overnight (18 hours) in a shaking incubator

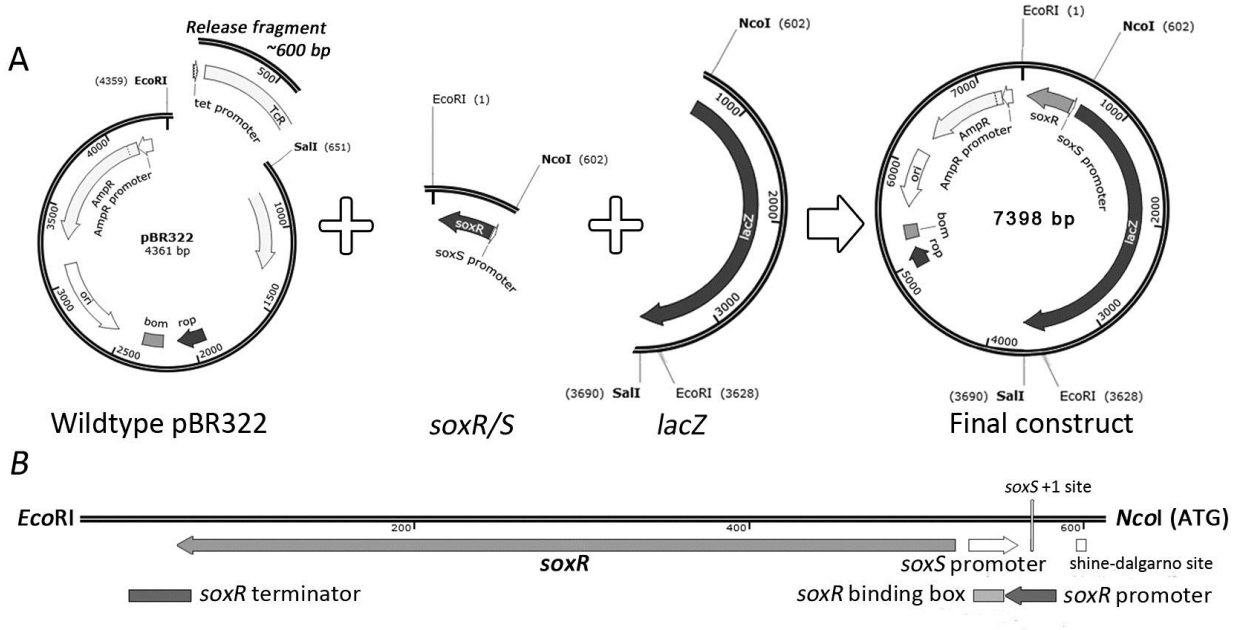

FIG 1. Cloning strategy. (a) pBR322 was first cut with *Eco*RI and *Sal*I to excise and inactivate the Tetracycline resistance gene. *soxR::*P_{soxS} and *lacZ* was then PCR amplified from *E. coli* MG1655 genome with the generated restriction sites. A 3-way ligation joined all the pieces together at the compatible sticky ends to form a 7.4 kb construct. *SoxR* homodimers in the host cell cytoplasm would detect internal ROS and activate *soxS*, which would then drive transcription of *lacZ*. (b) A close-up view of the cloned *soxR* fused to the *soxS* promoter, including the engineered restriction sites, the *soxR* regulation binding site and *soxS* native Shine-Dalgarno site.

at 37°C at 200 RPM. Growth in broth was measured as optical density (OD_{660}).

Plasmid selection, isolation, and primer construction. *E. coli* MG1655 genomic sequences were obtained from www.ecocyc.com and primers were manually designed to match genomic *lacZ* and *soxR::*P_{soxS} sequences. *Eco*RI and *Sal*I restriction sites were added onto the 5' end of the forward and reverse primers, respectively. Primers were ordered Integrated DNA Technologies (IDT) for synthesis. pBR322 plasmid was isolated from *E. coli* DH5α host strain from the MICB 421 culture collection and also from Invitrogen, supplied at 500 ng/µl. We grew the strains overnight and used the GeneJET Plasmid Purification System (Thermo Scientific) to extract pBR322 for use.

TABLE 1. 5'→3' Sequences of forward and reverse primers used to amplify *soxR::*P_{soxS} and *lacZ* from *E. coli* MG1655 genomic DNA. Engineered restriction sites are underlined and are flanked at the 5' end by 5 extra basepairs to allow for restriction enzyme recognition and cleavage.

Gene	Forward Primer	Reverse Primer
lacZ	5' - GGCGCCCATGGCT ATGACCATGATTA CG – 3'	5' – CCGGCGTCGACTT ATTTTTGACACCA GACC - 3'
soxR:: PsoxS	5' - CGGCGGAATTCGA GAAAGACAAAGAC CGGA - 3'	5' – GCGGCCCATGGAT CTGCCTCTTTTCAG TGT – 3'
soxR:: PsoxS and partial *lacZ*	5'- AAGGGAATAAGGG CGACACG – 3'	5' – TGTAAAACGACGG CCAGTGA- 3'

Polymerase chain reaction amplification of *soxR::*P_{soxS} and *lacZ*. To amplify the *soxR::*P_{soxS} and *lacZ* genes needed to make our construct, we used polymerase chain reaction (PCR) on *E. coli* strain MG1655, known to have a full *lac* operon and *soxR::*P_{soxS} gene. We extracted genomic DNA from *E. coli* MG1655 using DNeasy kit (Qiagen) kit. The PCR conditions were found to be the same for both the *soxR::*P_{soxS} and *lacZ* amplification. We used final concentrations of 1X PCR Reaction Buffer (Invitrogen), 2 units of Taq polymerase (Thermo scientific), 2.5 mM of $MgCl_2$ (Fermentas), 200 µM of dNTP (Invitrogen), 1 µM of primer from IDT of each forward and reverse primers, 50 ng to 100 ng of *E. coli* MG1655 genomic DNA template, and sterile distilled water were added to bring the PCR reaction up to a final volume of 25 µl. The amplification times were to first denature the DNA at 94°C for 5 minutes, then repeat the following steps 35 times: 94°C for 45 seconds, 64°C for 45 seconds, 72°C for 3.5 minutes for *lacZ* or 1.0 minute for soxR::P_{soxS}, and a final extension at 72°C at 10 minutes for both *lacZ* and *soxR::*P*soxS*. After PCR amplification, the PCR products were loaded into a 1.2 % agarose gel (UltraPure agarose select agar, Invitrogen) with 6X loading dye (Thermo scientific) and run for 45 minutes at 120 Volts. Products were visualized by soaking the agarose gel in an ethidium bromide bath (0.5 µg/ml) for 15 minutes. Later, 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.

Sequencing of PCR products. *lacZ* and *soxR::*P_{soxS} PCR products were sequenced using the protocol provided by Genewiz. We made a pre-mixed tube containing PCR products and primers with final concentrations of 20 ng/µl for *soxR::*P_{soxS} and 60 ng/µl for *lacZ* PCR products with 25 pmol of forward primers in a 15 µl tube before sequencing the PCR fragments with Genewiz. Analysis of sequencing data was performed using Snapgene Viewer (www.snapgene.com).

Purification of PCR amplified fragments and digestion. Successfully amplified *soxR::*P_{soxS} and *lacZ* PCR products were purified using a PCR purification kit (Purelink PCR Purification Kit, Invitrogen). Concentrations and purity of isolated PCR

products were determined by a Nanodrop spectrophotometer (Thermo scientific, Nanodrop 2000c spectrophotometer). Using the restriction enzymes *Eco*RI (GibcoBRL, 10 U/µl), *Sal*I (Invitrogen, 15 U/µl), and *Nco*I (New England Biolabs, 10 U/µl), and 10X REact 3 buffer (GibcoBRL), we double digested amplified PCR products and purified pBR322. All digestions had the same conditions: 1 µl of each restriction enzyme, 1 µg of DNA, 10X REact 3 was added to a final concentration of 1X REact 3 buffer and sterile distilled water was added to adjust the final volume to 25 µl. *soxR::*P_{soxS} was double digested with *Eco*RI, and *Nco*I, *lacZ* was digested with *Sal*I, and *Nco*I, and pBR322 was digested with *Eco*RI and *Sal*I. All of the digestions were carried out for 60 minutes at 37°C and followed by heat inactivation at 80°C for an additional 20 minutes to inactivate the restriction enzymes. The digested PCR products were purified using a PCR purification kit (Purelink PCR Purification Kit, Invitrogen) before the ligation reactions. 1 µg of digested pBR322 was run on a 1.2% agarose gel at 120 Volts for 45 minutes for gel purification (GenElute Gel Extraction Kit, Sigma-Aldrich).This purified, digested pBR322 was purified further with a PCR purification kit (Purelink PCR Purification Kit, Invitrogen) to remove trace amounts of agarose that the gel-purification kit failed to remove.

Ligation of digested PCR fragments and pBR322. We first carried out a three-way ligation by adding 100 ng of pBR322, 237 ng of *lacZ*, and 46 ng of *soxR::*P_{soxS} to the ligation mixture for a 1:3:3 molar ratio of pBR322, *lacZ* and *soxR::*P_{soxS} respectively. We also attempted ligation mixtures at a 1:6:6 molar ratio of vector to insert to insert. Required volumes of the digested products were added to the ligation mixture, along with 1 µl of T4 DNA Ligase (Fermentas, 5 Weiss U/µl), required volume of 10X Buffer for T4 DNA Ligase (Fermentas) needed to have a final concentration of 1X Buffer for T4 DNA Ligase, and sterile distilled water was added if needed to adjust the volume. The ligation reaction was incubated overnight (18 hours) at 16 °C. Ligations involving gel purified pBR322 were prepared as follows: 10 ng of gel purified pBR322, 5 ng of *soxR::*P_{soxS} and 25 ng of *lacZ* was added at a 1:3:3 ratio, along with 5 Weiss units of T4 ligase and 1X ligation buffer in a total volume of 10 µl. The ligation reaction was left to incubate for 2 hours at room temperature and then used to transform, and the remaining reaction was left overnight at 4°C and used to transform *E. coli* DH5α the next day.

Preparation of *E. coli* DH5α competent cells. Competent *E. coli* DH5α were prepared as described by Sambrook et al. with minor modifications (16). 1 ml of an overnight *E. coli* DH5α culture was prepared in LB at 37°C in a shaking incubator. The 1 ml overnight culture was inoculated into a 29 ml of LB in an Erlenmeyer flask until the OD_{660} reached a value of 0.4. Then cells were centrifuged at 3000 rpm for 10 minutes. The supernatant was removed and pelleted cells were then resuspended in 4 ml of sterile 0.1 M $CaCl_2$. The cells were then incubated on ice for 30 minutes and pelleted again under the same conditions. Finally, the cells were resuspended in 2 ml of 0.1 M $CaCl_2$ with 10% (w/v) glycerol and stored in -80°C until needed.

Transformation of *E. coli* DH5α $CaCl_2$ competent cells and heat shock. Competent cells were transformed using a modified heat shock protocol reported by Nogard et al. (17). To ensure transformation worked, cells were transformed with either 25 ng of pBR322 (Invitrogen) or 6 µl of the ligation mixture. DNA was mixed with the competent cells and the mixtures were incubated at 0°C on ice for 30 minutes, 45°C for 45 seconds and 0°C for 1 minute. The cells were recovered in LB for 30 minutes before plating onto LB agar plates and LB agar plates with final concentration of 100 µg/ml ampicillin. Transformants were incubated overnight (18 hours) at 37°C. The following day, transformants that grew on LB and ampicillin were replica plated onto LB and tetracycline plates with final concentration of 15 µg/ml.

Preparation of LB, LB plates, LB and ampicillin plates, LB, tetracycline plates and also LB plates with X-Gal, IPTG and ampicillin. The LB was prepared using the instructions in Difco & BBL Manual of Microbiological Culture Media, using 5 g of pancreatic digest of casein (Bacto Casaminoacids Technical, BD), 2.5 g of yeast extract (Difco yeast extract, BD), 0.25 g of sodium chloride (EMD chemicals Inc.) and 500 ml of sterile distilled water (15). The LB was autoclaved to ensure sterility before use. LB agar plates were prepared with the same protocol as LB, but also included 7.5 g of agar (Select agar, Invitrogen) before autoclaving and pouring into plates (15). Ampicillin, tetracycline, X-Gal, IPTG and ampicillin plates were prepared using directions from various laboratory manuals (16,18). Ampicillin, tetracycline and IPTG came in powdered stock forms, and were dissolved in water to the appropriate concentration. X-Gal came in a powdered stock form and was dissolved in tetramethylbenzidine. Antibiotics were added to liquid LB agar before plates were made, and X-Gal and IPTG were spread on the plates after they solidified. Ampicillin (Sigma-Aldrich) plates were prepared to have a final concentration of 100 µg/ml, tetracycline plates (Sigma-Aldrich) had a final concentration of 15 µg/ml, and LB agar plates with X-Gal (Fermentas) and IPTG (Sigma-Aldrich) and ampicillin had final concentrations of 100 µl of 2% X-Gal and 20 µl of 20% IPTG per plate, and 100 µg/ml, respectively (18).

β-galactosidase assay of transformants. Transformants that grew on ampicillin were analyzed for β-galactosidase activity using a β-galactosidase assay. All colonies along with positive controls (*E. coli* MG1655) were analyzed. All colonies were inoculated into a 1 ml LB in 10 ml tube and were grown overnight (18 hours) in a shaking incubator at 37°C. The following day, the cells were induced with or without paraquat (final concentration of 0.1 mM). The β-galactosidase assay was performed according to the protocol outlined by Sambrook et al.with slight modification (16). First 200 µl of toluene was added to 1 ml of fresh overnight culture, and cells were vortexed for 30 seconds. Then the bottom layer was removed and we added 1.2 ml of Tris-HCl (pH 8.0, 10 mM). Later 200 µl of *ortho*-nitrophenyl-β-galactoside (ONPG, 5 mM) was added and mixtures were incubated for 15 minutes at room temperature. Finally 2 ml of $NaHCO_3$ (0.6 M) was added and a visual confirmation for β-galactosidase activity was used. ONPG (5 mM) was prepared by dissolving ONPG at a concentration of 4 mg/ml in 0.1 M sodium phosphate as directed in Sambrook et al. (16).

RESULTS

PCR optimization was required to successfully amplify the *soxR::*P_{soxS} and *lacZ* region. Gradient PCR with annealing temperatures ranging from 55.0°C - 64.8°C produced desired amplification of *soxR::*P_{soxS} at approximately 600 bp and *lacZ* at approximately 3100 bp. However, the amplification of *lacZ* also resulted in amplification of non-specific products that decreased with increasing annealing temperature. Even at the higher annealing temperatures of 63.0°C and 64.8°C a second band at approximately 1600 bp remained (data not shown). Subsequent PCR reactions involved optimizing the PCR reaction mixture as well as the annealing temperature. Adjusting the annealing temperature to 64.0°C, and doubling the amount of genomic DNA and Taq polymerase resulted in successful amplification of *lacZ*

without any non-specific products (Fig. 2). Successful amplification of the *soxR::*P_{soxS} and *lacZ* region is shown in Figure 2, with *soxR::*P_{soxS} at approximately 600 bp, and *lacZ* at approximately 3100 bp in lanes 1-2, respectively. Negative controls without added DNA template were shown in lanes 3-4 in which no amplification was observed.

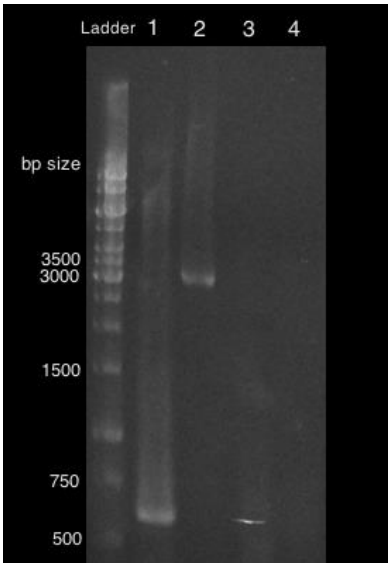

FIG 2. Optimized PCR amplification of *soxR::*P_{soxS} and *lacZ*, and negative controls.Lane 1 and 2 contains *lacZ* and *soxR::*P_{soxS} PCR products respectively, using *E. coli* MG1655 genomic DNA as the template. Lanes 3 and 4 contain no added template using *soxR::*P_{soxS} and *lacZ* primer sets, respectively. GeneRuler^{TM} 1kb DNA Ladder was used.

Sequencing of purified PCR products confirmed the amplification of the correct *soxR::*P_{soxS} sequence with high sequence similarity (data not shown). The sequence for *lacZ* was of poor quality, with only 59 bp with Phred scores higher than 25; however, 24 of these basepairs matched the 5' end of the *lacZ* sequence, suggesting that we had *lacZ*.

Digestion and purification *of soxR::*P_{soxS} and *lacZ* resulted in low yields. Amplification of *soxR::*$P_{soxS}$and *lacZ* were successful in yielding relatively high amounts of DNA; however purification of *lacZ* consistently resulted in considerable reductions in concentration in comparison to its pre-purified state (data not shown). Furthermore, purification of the *soxR::*P_{soxS} and *lacZ* following double restriction enzyme digestion of *soxR::*P_{soxS} with *Eco*RI and *Nco*I, and of *lacZ* with *Nco*I and *Sal*I in order to eliminate interfering release fragments and enzymes before ligation ultimately resulted in no DNA return from both. Consequently, all subsequent digestion reactions of *soxR::*P_{soxS} and *lacZ* remained unpurified and samples were directly used in proceeding ligation reactions.

The digestions of *soxR::*P_{soxS} and *lacZ* fragments were presumed to be successful considering that the enzyme activities of the various restriction endonucleases were confirmed using λ DNA. λ DNA digested with *Nco*I, *Eco*RI, and *Sal*I sequentially, and double digestion resulted in multiple fragments when ran on an agarose gel while the untreated DNA remained unfragmented, thus verifying functional enzyme activity of the endonucleases required to cut the *soxR::*P_{soxS} and *lacZ* (data not shown).

Digestion and gel purification of pBR322 vector plasmid was successful and was confirmed by running the undigested and digested sample on an agarose gel. As expected, the released fragment appeared at approximately 650 bp, while the linearized pBR322 vector plasmid appeared at 4000 bp (data not shown). The gel-purified product of the linearized pBR322 vector appeared at the equivalent base pair size, without showing any release fragment consistent with a successful purification procedure. However, the higher mass region of the ladder was not fully resolved and so an accurate size estimation of the linearized pBR322 fragment could not be inferred (data not shown).

Unsuccessful acquisition of recombinant pBR322 vector plasmid containing ligated *soxR::*P_{soxS} and *lacZ* fragments. Ligations were performed using gel-purified or unpurified digested pBR322 and subsequently used for transformation. Transformants were replica plated onto LB agar plates containing ampicillin and LB agar plates containing tetracycline for selection. Successful transformants with the desired recombinant plasmid were expected to maintain ampicillin resistance while conferring sensitivity to tetracycline. However, transformants using gel-purified digested pPBR322 resulted in no viable colonies, while all colonies of transformants using unpurified digested pBR322 grew on both selective plates. *E. coli* strains DH5α containing pBR322 and MG1655 used as controls showed the expected growth characteristics on ampicillin and tetracycline. As predicted, *E. coli* DH5α containing pBR322 was able to grow on both selected plates while *E. coli* MG1655 did not grow.

To ensure the use of appropriate strains used during the process of constructing the constituent pieces of the *soxR::*P_{soxS} and *lacZ* fusion reporter, as well as accounting for the function of β-galactosidase and the potential inducibility in its native form as part of the full *lac* operon, various strains, along with two of the transformant colonies were plated onto LB agar containing IPTG and X-gal. Transformants that grew on both ampicillin and tetracycline, transformation competent *E. coli* DH5α, and *E. coli* MG1655 all exhibited growth, however β-galactosidase activity was only observed in *E. coli* MG1655. These results suggest proper strains were used during the preparation of the constituent parts of the promoter fusion construct, while the transformants displayed no indication of acquiring the construct.

To verify the absence of the construct, purified plasmid extractions from transformants were linearized by digestion with *Pst*I while PCR reactions were carried out using the same plasmid extractions, as well as from *E. coli* MG1655 genome as a positive control to confirm for the presence or absence of *soxR::*P_{soxS} and *lacZ*. As expected, the linearized plasmids from colonies 3, 7, 9, 11 and 13 showed faint bands at approximately 4300 bp (Fig. 3).

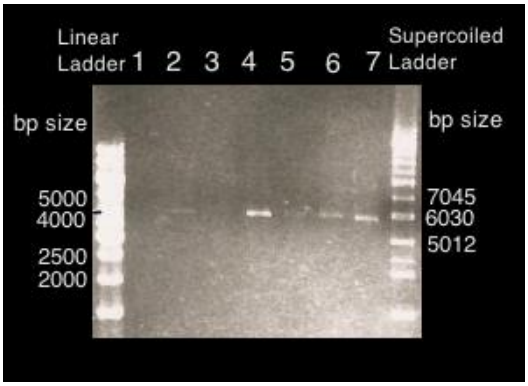

FIG 3. Transformant plasmid extractions exhibiting undesired plasmid size. Lanes 1-7 contain *Pst*I digested plasmids of various transformant colonies that grew on ampicillin. GeneRuler™ 1 kb DNA Ladder and Invitrogen™ Supercoiled DNA Ladder was used, respectively.

The PCR reactions showed amplifications of both *soxR::*P_{soxS} and *lacZ* in the selected colonies, (Fig. 4) suggesting the presence of *soxR::*P_{soxS} and *lacZ* in the plasmids. To eliminate the possibility of contamination by genomic DNA, mock plasmids from *E. coli* strains DH5α and MG1655 were isolated and PCR amplified for the presence of *lacZ*. As anticipated, no amplification occurred, as evidenced from an agarose gel (data not shown).

An assay for β-galactosidase using ONPG as a substrate was performed on all transformants that grew on ampicillin plates. Negative results for β-galactosidase activity were seen in all of these colonies, as well as in the controls: *E. coli* transformation competent DH5α, *E. coli* DH5α containing pBR322, and LB negative control. Positive β-galactosidase activity was only seen in the positive controls of *E. coli* MG1655, and *E. coli* MG1655 under paraquat induction. An additional assay was done to confirm the lack of ligated *soxR::*P_{soxS} and *lacZ* in the recombinant plasmids by PCR amplification using primers (confirmation primers) that were designed to amplify the full *soxR::*P_{soxS} region along with part of the *bla* gene. The gels displayed significant non-specific amplification while showing no distinct bands representative of partial pBR322 vector plasmid, full *soxR::*P_{soxS} and partial *lacZ* gene region that was to be amplified, which would have corresponded to approximately 900 bp (data not shown). Thus, the acquisition of a recombinant plasmid containing the proper *soxR::*P_{soxS}*::lacZ* reporter construct by transformant colonies was not achieved.

Investigation into the lack of successful transformants found that ligation was unsuccessful. Gel electrophoresis analysis of the ligation reaction between *soxR::*P_{soxS} and *lacZ* did not show a fragment of approximately 3700 bp, which would be expected if successful ligation between *soxR::*P_{soxS} and *lacZ* did occur. Instead, the gel displayed a low molecular weight smear (data not shown). Subsequently, PCR was performed using forward *soxR::*P_{soxS} primer with *lacZ* reverse primer to look for the presence of the 3700 bp product that would indicate the presence of a successful ligation between *soxR::*P_{soxS} and *LacZ*. However, this PCR did not yield successful products.

Finally, PCR was performed to verify the presence of properly ligated sequence using the ligation reaction mixture as template. Multiple reactions were run using the various primers: *soxR::*P_{soxS} forward with *soxR::*P_{soxS} reverse, *lacZ* forward with *lacZ* reverse, and the set of confirmation primers, designed to amplify *soxR::*P_{soxS} and part of *lacZ*. It was expected that with proper ligation products as template, the gel would show the presence of amplified ***soxR::***P_{soxS} at 600 bp, *lacZ* at 3100 bp, and 900 bp for the full *soxR::*P_{soxS} and partial *lacZ* gene. However, contrary to the anticipated outcome, all samples resulted in smears (data not shown). As negative controls for the reactions involving the confirmation primers, *E. coli* MG1655 genomic DNA and pBR322 plasmid was also run using the same primers. While some smearing was present in both samples, a few distinct bands were present but none appeared at approximately 900 bp to indicate the existence of the partial *soxR::*P_{soxS}::lacZ fragment as presumed.

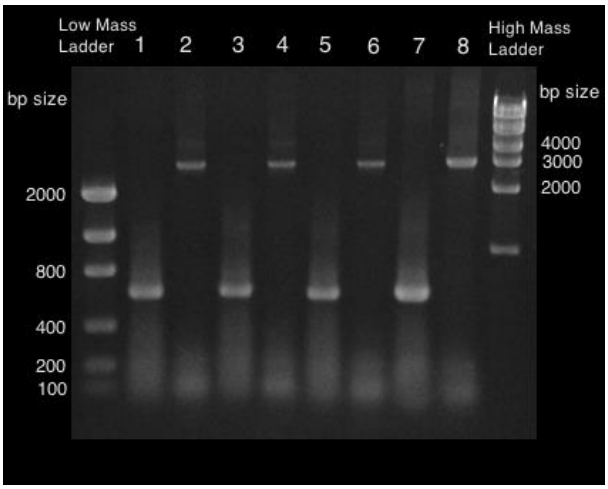

FIG 4. **Transformant plasmid extractions assessing the presence of *soxR::*P_{soxS} and *lacZ***. PCR amplifications using *soxR::*P_{soxS} primer set and transformant plasmids (lanes 1,3,5), or *E.coli* MG1655 (lane 7) as a template. Lanes 2, 4, 6 and 8 contain PCR amplifications using *lacZ* primer set of transformant plasmids and *E. coli* MG1655 respectively. Low DNA Mass™ Ladder and Invitrogen™ High DNA Mass Ladder were used, respectively.

In a final attempt to obtain the desired construct, we performed a 3-way ligation using *soxR::*P_{soxS}*,lacZ,* and gel-purified digested pBR322 and transformed cells. None of the transformants exhibited the differential antibiotic resistance that would indicate the acquisition of the desired recombinant plasmid. These results showed that there is strong evidence that the *soxR::*P_{soxS} and *lacZ* fragments failed to ligate to one another, and ultimately the construction of a *soxR::*P_{soxS}*::lacZ* sensitive ROS reporter was not obtained.

DISCUSSION

We attempted to construct a reporter plasmid, containing *soxR::*P_{soxS} and *lacZ.* This plasmid would allow for the detection of internal ROS by SoxR, which would then bind to the *soxS* promoter and upregulate *lacZ*. We successfully PCR amplified *lacZ*, and *soxR* fused to P_{soxS}. We also confirmed that our restriction enzymes were able to cut, and we were also able to gel-purify digested pBR322, the vector backbone for our construct, to remove the approximately 600 bp release fragment. Despite having all the working parts, we could not successfully ligate the pieces together to achieve a complete construct.

We successfully PCR amplified *lacZ* from *E. coli* MG1655 as confirmed by size and supported by sequencing data; however, we also obtained non-specific products at lower annealing temperatures, which disappeared at higher temperatures above 64°C (data not shown). Krentz previously reported that PCR amplification of *lacZ* from *E. coli* was unsuccessful, and instead she obtained non-specific products at 1500 bp and 700 bp in size using a different set of primers (19). In contrast, we successfully PCR amplified pure *lacZ* without non-specific products by designing different primers and increasing the annealing temperature; therefore, our study showed that amplifying genomic *lacZ* from *E. coli* was possible. Future studies requiring *lacZ* should use the primers and reaction conditions specified in this report.

When we ligated using digested pBR322 without gel-purifying it and transformed the ligation reactions into competent cells, we obtained many colonies that were all tetracycline and ampicillin resistant. These resistant colonies may have arisen from a small amount of pBR322that was not digested and subsequently selected for after transformation. When we removed the released fragment by gel-purifying digested pBR322 backbone and used this in the ligation reaction, we were unable to obtain any colonies. The disappearance of previously observed double-resistant colonies may have been due to the removal of undigested pBR322 by gel purification Alternatively, because only 1 ng/µL of digested and gel-purified pBR322 backbone DNA was used for this ligation and transformation (to maintain proper 3:3:1 ratio due to limiting concentrations of lacZ), it is possible that there was not enough DNA to successfully transform bacteria and obtain viable colonies.

It is particularly strange that when we performed PCR reactions using the plasmids from ampicillin and tetracycline double resistant colonies' as template, we unexpectedly obtained both *lacZ* and *soxR::*P_{soxS} fragments (Fig. 4). Thinking this may have been genomic DNA contamination carried over from the plasmid extraction, we also performed PCR on "mock extracted" plasmids, to ensure purification indeed removed all genomic contaminants. This was done by putting *E. coli* strains DH5α containing pBR322 and MG1655 (lacks plasmid) through the plasmid purification kit and using the resulting sample as template – we saw no *lacZ* nor *soxR::*P_{soxS} bands, ruling out contamination from the kit. Because digested pBR322, *soxR::*P_{soxS} and *lacZ* fragments were mixed together for the ligation reaction, it could have been possible for pBR322 to re-ligate with its release fragment and/or ligate with the components of our construct. In this case, perhaps both pBR322 and a successful recombinant plasmid were taken up by the cells simultaneously, which is known to happen (21). However, the reason we could not see our 8 kb construct when we ran the extracted plasmids on a gel (Fig. 3) may be due to our construct not being able to replicate efficiently, given the large size of the construct. It has also been previously reported that efficient transcription of cloned genes in pBR322 may reduce the plasmid's replication efficiency (22). In our case, perhaps *soxR* but not *lacZ* was highly and efficiently transcribed because LacZ activity was not detected by β-galactosidase assay. Coupled with pBR322 intrinsically being a medium copy number plasmid, our construct's replication may have been significantly hampered by *soxR* transcription. This may also explain the lack of colonies when we transformed using gel-purified pBR322 as backbone because transcription of the antibiotic resistance genes on the plasmid may also be hampered by the *soxR* expression. Furthermore, this phenomenon may also explain the lack of β-galactosidase activity detected by the β-galactosidase assay. Although these explanations are sufficient to explain our results, the probability that all three of our tested colonies randomly contain both plasmids is remarkably low and therefore there may be another reason that a PCR done on plasmids isolated from colonies that grew on ampicillin and tetracycline showed *lacZ* and *sox* products despite them being absent.

In order to obtain a successful construct, our 3-way ligation must be successful: all three of the *Nco*I, *Eco*RI, and *Sal*I digested sites must ligate. Even if one of the three sites failed to ligate, we would not have had a working construct and thus no transformants. Previous reports have shown that *Sal*I digested fragments have low ligation efficiency (23,24). Perhaps the lack of a ligated construct, and thus the lack of transformants, is due to the low efficiency of the *Sal*I site ligation.

Another possible reason for the low efficiency of ligation could be due to the improper molar ratios of insert and vector used for ligation. In general, ligation reactions involve an excess of insert to vector (25). Specifically, Invitrogen recommends a 3:1 molar ratio of insert to vector (26), and previous Journal of Experimental Microbiology and Immunology reports have used this ratio (27). Our experiments in this study

used a 3-way ligation, and we attempted a 3:3:1 and 6:6:1 molar ratio of *soxR::*P_{soxS}*::lacZ::*pBR322, but neither worked. Furthermore, the amount of backbone pBR322 DNA used for ligations can also vary significantly. Successful ligations report using from 80 ng to 1000 ng, but the volumes were not indicated so concentrations could not be calculated (23,27). In this report, we attempted 1 and 2 ng/µl of pBR322 backbone and corresponding molar amounts of insert. Further manipulation and optimization of backbone concentration and vector to insert ratios may yield successful transformants.

In conclusion, we successfully produced all the working parts of our desired construct, but failed to successfully obtain our desired transformant. This may be due to two reasons: the *soxR::*P_{soxS} gene may hamper plasmid replication and expression and therefore the construct is not maintained within transformants, or the lack of a successful construct may be due to failure to ligate, and the *soxR::*P_{soxS} and *lacZ* PCR results using plasmid from ampicillin and tetracycline resistant colonies may have been due to *E. coli* MG1655 genome contamination. Further manipulation of ligation variables utilizing gel-purified pBR322 as the vector backbone should be tested before ruling out the effectiveness of the proposed construct.

FUTURE DIRECTIONS

It is clear from our studies that optimization of ligation reactions must first occur to create the recombinant plasmid. Increasing the DNA concentrations used, particularly the concentrations of the *lacZ* fragments in the reactions would help enhance the reaction efficiency. Furthermore, it would be useful to examine the effects of using various ratios of insert to vector plasmid to improve the likelihood that the overhang ends of the larger molecular-weight fragments will encounter the proper counterpart. Other methods can be used to increase probability of generating the desired construct, such as the addition of Polyethylene glycol to the ligation mixture, or PCR amplification of correctly ligated constructs using multiple displacement amplification (28,29). Using the electroporation method may increase the transformation efficiency and increase the yield of colonies to improve the chance of finding the proper clone (30). Upon constructing and verifying the presence of the proper recombinant plasmid (through a β-galactosidase assay and sequencing the plasmid), the functionality and the limitations of the reporter system should be tested. Given that *E. coli* DH5α still contains the native *sox* regulon, sequestrations of the SoxR regulator may occur and interfere with expression of *lacZ* on the plasmid. Thus, considerable induction under paraquat may be necessary for the observable expression and enzymatic activity of β-galactosidase. Future experiments can attempt to optimize the various ligation reaction parameters, and subsequently analyze the functionality of the *soxR::*P_{soxS}*::lacZ* reporter construct.

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REFERENCES

1. **Brem R, Li F, Karran P**. 2009. Reactive oxygen species generated by thiopurine/UVA cause irreparable transcription-blocking DNA lesions. Nucleic Acids Res. **37**: 1951-1961.
2. **Bus JS, Gibson JE**. 1984. Paraquat: model for oxidant-initiated toxicity. Environmental Health Perspectives. **55**:37-46.
3. **Dalton TP, Shertzer HG**, Puga A. 1999. Regulation of gene expression by reactive oxygen. Annu. Rev. Pharmacol. Toxicol. **39**:67-101.
4. **Cabiscol E, Tamarit J, Ros J**. 2000. Oxidative stress in bacteria and protein damage by reactive oxygen species. Int. Microbiol. **3**:3-8.
5. **Belkin S, Smulski DR, Vollmer AC, Van Dyk TK, LaRoussa RA**. 1996. Oxidative stress detection with *Escherichia coli* harboring a *katG'::lux* fusion. Appl. Environ. Microbiol. **62**:2252-2256.
6. **Lushchak VI**. 2001. Oxidative stress and mechanisms of protection against it in bacteria. Biochemistry. **66**:476-489.
7. **Hoerter JD, Arnold AA, Kuczynska DA, Shibua A, Ward CS, Sauer MG, Gizachew A, Hotchkiss TM, Fleming TJ, Johnson S**. 2005. Effects of sublethal UVA irradiation on activity levels of oxidative defense enzymes and protein oxidation in *Escherichia coli*. J. Photochem. Photobiol. **81**:171-180.
8. **Eisenstark A, Perrot G**. 1987. Catalase has only a minor role in protection against near-ultraviolet radiation damage in bacteria. Mol Gen Genet. **207**:68-72.
9. **Gordon R, Livingstone B, Pho V**. 2011. Transcription of *katG* is enhanced in *Escherichia coli* exposed to UV-A and might enhance cell survival. J. Exptl. Microbiol. Immunol. **15**:111-116.
10. **Greenberg JT, Monach P, Chou JH, Josephy PD, Demple B**. 1990. Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. PNAS. **87**:6181-6185.
11. **Pomposiello PJ, Demple B**. 2001. Redox-operated genetic switches: the SoxR and OxyR transcription factors. Trends Biotechnol. **19**:109-114.
12. **Partridge JD, Scott C, Tang Y, Pool RK, Green J**. 2006. *Escherichia coli* transcriptome dynamics during the transition from anaerobic to aerobic conditions. J. Biol. Chem. **281**:27806-27815.
13. **Jain VK, Magarth IT**. 1991. A chemiluminescent assay for quantitation of β-galactosidase in the femtogram range: Application to quantitation of β-galactosidase in *lacZ*-transfected cells. Analytical Biochemistry. **199**:119-124.
14. **Friedrich WF, Greenberg EP**. 1983. Glucose Repression of Luminescence and Luciferase in *Vibrio fischeri*. Arch. Microbiol. 134:87-91.
15. **Becton, Dickinson and Company.** 2003. Difco & BBL Manual, Manual of Microbiological Culture Media. Sparks, Maryland. 318.
16. **Sambrook J, Fritsch EF, Maniatis T.** 1989. Molecular Cloning: A Laboratory Manual, Vol. I. 2nd Edition. Cold Spring Harbor Laboratory Press, NY. 17.50-17.51.
17. **Nogard MV, Keem K, Monahan JJ.** Factors affecting the transformation of *Escherichia coli* strain χ1776 by pBR322 plasmid DNA. Gene. **4**:353-354.
18. **Barker, Kathy.** 2005. At the Bench: A Laboratory Navigator. Cold Spring Harbor Laboratory Press, NY. 257-258.

19. **Krentz C.** 2004. Polymerase chain reaction amplification of *lacZ* to engineer a gene sequence that can be used to modify the size of pUC19. J. Exp. Microbiol. Immunol. **6:**35-38.
20. **Kuzminov A, Schabtach E, Stahl FW.** 1994. Chi sites in combination with RecA protein increase the survival of linear DNA in Escherichia coli by inactivating exoV activity of RecBCD nuclease. EMBO J. **13:**2764-2776.
21. **Goldsmith M, Kiss C, Bradbury AR, Tawfik DS.** 2007. Avoiding and controlling double transformation artifacts. Protein Eng Des Sel. **20:**315-318.
22. **Stueber D, Bujard H.** 1982. Transcription from efficient promoters can interfere with plasmid replication and diminish expression of plasmid specified genes. EMBO J. **1:**1399–1404.
23. **Bola G.** 2005. Evaluating the role of G,C-nucleotides and length of overhangs in T4 DNA Ligase efficiency. J. Exp. Microbiol. Immunol. **8:**1-7.
24. **Dhesi S.** 2005. Investigation of the relationship between terminal sequence variation and the ligation efficiency of T4 DNA Ligase on blunt-ended DNA. J. Exp. Microbiol. Immunol. **8:**14-20.
25. **Ish-Horowicz D, Burke JF.** 1981. Rapid and efficient cosmid cloning. Nucleic. Acids Res. **9:**2989-2998.
26. **Invitrogen Corporation.** 2002. T4 DNA ligase technical bulletin 15244-2. Invitrogen, Carlsbad, CA.
27. **Chow P.** 2005. Cloning of λ DNA fragments into pUC19 vector to study the ligation efficiency of *Nde*I-digested pUC19 and *Hind*III-digested pUC19 by T4 DNA ligase. J. Exp. Microbiol. Immunol. **8:**8-13.
28. **Pheiffer BH, Zimmerman SB.** 1983. Polymer-stimulated ligation: enhanced blunt- or cohesive-end ligation of DNA or deoxyribooligonucleotides by T4 DNA ligase in polymer solutions. Nucleic Acids Res. **11**:7853-7871.
29. **Christ D, Famm K, Winter G.** 2006. Tapping diversity lost in transformations - in vitro amplification of ligation reactions. Nucleic Acids Res.**34:**e108.
30. **Dower WJ, Miller JF, Ragsdale CW.** 1988. High efficiency transformation of E. coli by high voltage electroporation. Nucleic Acids Res. **16:**6127-6145.