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Attempted Transformation of Catalase-Deficient *Escherichia coli* with pBAD24 Vector Containing *katE*

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A pBAD24 vector containing *katE* was constructed for use in understanding the effect of *katE* expression on the UVA-mediated killing of catalase-deficient *E. coli*. PCR amplified *katE* was cloned into the pBAD24 expression vector and transformed by electroporation into the catalase-deficient UM2 strain. Screening by colony PCR and digestion of isolated plasmids yielded no positive transformants. Since agarose gel electrophoresis supports successful construction, failure was likely attributable to outnumbering by other pBAD24 containing ligation products or host-construct incompatibility. We propose the metabolic load phenomenon, RecBCD mediated construct instability, and the EcoK restriction system as explanations for host-construct incompatibility.

SODIS (Solar Water Disinfection) is commonly used as an inexpensive means of killing harmful pathogens in drinking water by exposing water to prolonged periods of sunlight (19). Prolonged UV-A exposure mediates bacterial killing by increasing water temperature and generating reactive oxygen species (ROS) through UV-A absorption by endogenous or exogenous photosensitizers (12). *Escherichia coli* expression of two catalase genes, *katG* and *katE*, which respectively code for hydroperoxidase-1 (HP1) and hydroperoxidase-2 (HPII), help prevent the formation of damaging reactive oxygen species (ROS) (6, 9). Previous experiments assessing the importance of catalase in the context of UV-A disinfection of water found that the *E. coli* UM2 double mutant lacking *katE* and *katG* genes exhibited reduced survival after UV-A irradiation compared to both wild type *E. coli* and the *E. coli* UM197 single mutant strain lacking only *katG* (1). More recently, *katE* complementation of the UM2 double mutant strain was performed (4). Surprisingly, the complemented strain showed the lowest survival of all of the strains after UV-A exposure, even when compared to the UM2 double mutant and UM197 single mutant strains (4). As the plasmid used for the complementation was a M13 Bluescript KS' high copy number plasmid (500-700 copies/chromosome) (13), it was suggested that overexpression of *katE* was potentially responsible for the observed decrease in survival (4). Studies have noted that overexpression of endogenous catalase along with UV-A exposure causes the generation of ROS rather than the neutralization of such species (9).

In this study, we cloned the *katE* gene into the pBAD24 expression vector to better understand the consequences of catalase expression (8). The pBAD expression vectors are regulated using the P_{BAD} promoter and AraC (8). Gene transcription can be induced by the presence of L-arabinose (8). By using this construct, it's possible to control catalase expression in *E. coli* UM2 to assess the effect of a range of catalase expression levels on cell survival. Unfortunately, our attempts to achieve controlled *katE* expression were hindered by our inability to obtain a transformant containing our construct. The selection of methods used to obtain a clone can affect the probability of success. For the consideration of future studies in regulating *katE* expression or other cloning experiments, here we evaluate the methods used in our approach to explain our inability to obtain a transformant.

METHODS AND MATERIALS

Bacterial strains and plasmids. The plasmid pAM*katE*72 was used as the template for *katE* amplification. *E. coli* UM255 carrying pAM*katE*72 and *E. coli* UM2 [F-, *araC14*, *leuB6*(Am), *secA206*(aziR), *fhuA23*, *lacY1*, *proC83*, *tsx-67*, *purE42*, *glnV44*(AS), *galK2*(Oc), *LAM-*, *trpE38*, *xthA15*, *his-208*, *rfbC1*, *mgl-51*, *argG77*, *rpsL109*(strR), *glpR201*,*xylA5*, *mtl-1*, *ilvA681*, *thi-1*, *metA160*, *katG15*, *katE2*] were provided by the MICB 421 bacterial strain collection (Department of Microbiology and Immunology, University of British Columbia). The pBAD24 vector (4.5 kbp) was used to control *katE* expression. DH5α carrying pBAD24 was obtained from the Hancock laboratory (Department of Microbiology and Immunology, University of British Columbia).

Isolation of pAM*katE*72 and pBAD24. 5 ml Luria-Bertani (LB) broth [1% w/v tryptone (Bacto**;** Cat. No. 211701), 0.5% w/v yeast extract (Bacto; Cat. No. 212730), 1% w/v NaCl (Fisher; Cat. No. 642-500)] was inoculated with UM255 carrying pAM*katE*72 or DH5α carrying pBAD24 and incubated overnight at 180 RPM and 37°C. The pAM*katE*72 plasmid was isolated using the Purelink Quick Plasmid Miniprep Kit (Invitrogen; Cat. No. K210010). The pBAD24

vector was isolated using the GeneJET Plasmid Miniprep Kit (Fermentas; Cat. No S0502). Concentration and purity of both were determined by measuring absorbance at 260 nm and 280 nm with a spectrophotometer (Beckman DU 530).

Amplification of *katE*. The *katE* gene sequence was reported by Loewen *et al.* (17). Primer-BLAST (National Center for Biotechnology Information) was used to design primers to amplify a 2541 bp fragment encoding the *katE* ribosomal binding site, the open reading frame, and the transcription termination sequences. *Kpn*I restriction sites were added to their 5' ends for ligation into pBAD24. Two arbitrary nucleotides (5'-AT-3') were added to the 5' ends of the restriction sites for efficient cleavage. NEBcutter V2.0 (New England Biolabs) confirmed the *katE* fragment contained no *Kpn*I restriction sites. The following forward and reverse primers (*katE*-F, *katE*-R) were ordered from IDT technology (Coralville, IA): 5'-ATGGTACCAGCGGGATCTGGCTGGTGGT-3' and 5' ATGGTACCACTGCCGCCGCTTGAGACTG-3', respectively. The PCR contained 2 µl of 10X Taq Buffer +KCl -MgCl2 (Fermentas; Cat. No. B38), 1.5 mM $MgCl_2$ (Fermentas; Cat. No. #R0971), 0.8 mM dNTP mix (Fermentas; 25 mM Cat. No. #R1121), 0.2 µM forward primer, 0.2 µM reverse primer, 0.00125 U Taq polymerase (Fermentas; Cat. No. EP0402), and 49.2 ng of pAM*katE*. The PCR was performed using the Biometra T-gradient thermocycler programmed to a 95°C initial denaturation for 2 minutes, 40 cycles of 95°C for 30 seconds, 62.6°C for 30 seconds, and 72°C for 2.5 minutes, and a final elongation at 72°C for 5 minutes. PCR products were stored at 4°C. 1% agarose gel electrophoresis was used to confirm *katE* amplification. 1% agarose (Biorad Certified™ Molecular Biology Agarose Cat No 161-3101) gels were prepared with 1X TBE buffer. Gels were run at 100 V for 45-90 minutes in 1X TBE buffer (89 mM Tris Base (Fisher Scientific; Cat. No. BP152-1), 89 mM Boric Acid (Sigma; Cat. No. B0252), 2 mM EDTA, pH 8 (Fisher; Cat No. S-311). Samples were prepared for electrophoresis using 6X DNA Massruler loading dye (Fermentas; Cat No #R0621). GeneRuler™ 1 kb DNA Ladder, ready-to-use, 250-10,000 bp (Fermentas Cat. No. SM0314), was used to estimate linear fragment sizes. Gels were stained in 0.2 µg/ml ethidium bromide for 20-30 minutes after electrophoresis.

Cloning *katE* into pBAD24. *Kpn*I digestion of the *katE* PCR fragment was performed using 6 µl *katE* PCR product (403 ng/µl), 2 µl *Kpn*I (from 10 U/µl supply) (Invitrogen Cat No: 15232 010), 2 µl 10X REact 4 Buffer (Invitrogen; Cat. No. Y90005), and 10 µl dH_2O for a total reaction volume of 20 µl. *Kpn*I digestion of pBAD24 vector was performed using 20 µl isolated pBAD24 (52 ng/µl), 2 µl *Kpn*I (10 U/µl), 5 µl 10X REact 4 Buffer and 23 µl dH_2O for a total reaction volume of 50 µl. The *Kpn*I digests were incubated at 37°C for 1 hour followed by heat inactivation for 30 minutes at 65°C. The terminal 5' phosphate groups on the digested pBAD24 vector were removed by adding 1 µl (5 U) Antarctic Phosphatase (5000U/ml NE Biolabs Cat. No. M02895) and 5 µl 10X Antarctic Phosphatase Buffer (NE Biolabs; Cat. No. B02895). The dephosphorylation reaction was incubated at 37°C for 20 minutes followed by heat inactivation at 65°C for 8 minutes. Agarose gel electrophoresis confirmed cleavage by *Kpn*I. Ligation was done in 24 µl solution of 1.38 µl digested *katE* PCR fragment, 4.85 µl of the digested pBAD plasmid, 3.77 µl dH20, 2 µl of 5X T4 buffer (Fermentas; Cat. No. B69), 1 µl of T4 ligase (Fermentas; Cat. No. E10001). This reaction was run at 4°C overnight.

Making UM2 electrocompetent cells. Preparation of electrocompetent cells and electroporation were performed according to Bio-Rad's MicroPulser Electroporation Apparatus Operating Instructions and Applications Guide (Bio-Rad, #165-2100). 50 ml of LB broth was inoculated with 2.5 ml of fresh overnight *E. coli* UM2 culture. The cells were incubated at 37°C shaking at 180 rpm until they reached an OD_{600} of ~0.5, after which the culture was chilled on ice for 20 minutes. The culture was then split between two sterile 30 ml Oakridge centrifuge tubes and the cells were spun at 4000 x g for 15 minutes at 4°C. Pellets were washed twice with 20 ml ice-cold sterile distilled water. Each pellet was resuspended in 1 ml of ice-cold 10% glycerol, spun as before, and the supernatant was discarded. Each pellet was resuspended in 100 µl of ice cold 10% glycerol and the cell suspensions were combined. Aliquots of 40 µl of the cell suspension were frozen at -80°C.

Transformation of *katE*-pBAD24 construct into UM2 cells. One 40 µl aliquot each of UM2 and DH5α electrocompetent cells and two 0.2 cm Bio-Rad electroporation cuvettes were thawed on ice 15 minutes prior to electroporation. 2.7 µl of the ligation reaction (amount of DNA = 27 ng) was added to the UM2 cells. The mixtures were incubated on ice for ~1 minute and then transferred to the pre-chilled electroporation cuvettes. Each mixture was pulsed at 2.5 kV with a time constant of ~2.5 ms. Then, 1 ml of SOC media was immediately added to each mixture and the suspensions were transferred to 1.5 ml tubes. The cells were incubated at 37°C shaking at 180 rpm for 1 hour. 100 µl of 10^{-1}, 10^{-2}, and 10^{-3} dilutions were plated onto LB agar plates containing ampicillin (100 ug/ml) and L-arabinose (0.2%), and the plates were incubated overnight at 37°C.

***Sal*I digestion of pBAD24.** To test whether proper ligation occurred, *Sal*I digestions of our ligation reaction (Invitrogen; Cat. No. 52175A) were performed using 5 µl of our ligation reaction, 2 µl of either 10X Buffer O (Fermentas; B05) or 10X React 10 (Invitrogen; Y90012), 1 µl of *Sal*I (10U/µl), and 12 µl of sterile HPLC water for a total reaction volume of 20 µl. Both digestions were incubated at 37°C for 2 hours. Heat inactivation was done for 20 minutes at 65°C**.** The digestions were analyzed using agarose gel electrophoresis as before.

Isolation and *Kpn*I digestion of transformant plasmid DNA. To test whether UM2 cells had pBAD24+*katE*, sterile test tubes containing 5 ml of LB broth, L-arabinose (0.2%) (Aldrich; Cat. No. A91906-1006-A), and ampicillin (100 ug/ml) (Sigma; Cat. No. A9518) were inoculated with transformant colonies. The cultures were incubated at 37°C shaking at 200 rpm overnight. Plasmid DNA was extracted from 2 ml of each of the overnight cultures using the GeneJET kit as mentioned above. *Kpn*I digestion of isolated plasmid DNA was performed as mentioned above.

Screening PCR of UM2 colonies. Two PCR reactions using a single annealing temperature and gradient temperature were used to screen for the presence of the *katE* gene in the plasmid DNA of electroporated UM2 cells. Each PCR tube contained 1 µl of 10X Taq Buffer, 0.6 µl of 25 mM $MgCl_2$, 0.08 µl of 25 mM dNTP mix, 0.2 µl of 10 µM forward primer (*katE*-F), 0.2 µl of 10 µM reverse primer (pBAD24R), 0.00125 U Taq polymerase, and 7.87 µl of sterile water.

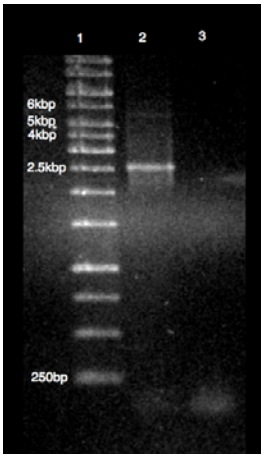

FIG. 1: PCR using pAM*katE*72 template to obtain *katE* with *Kpn*I restriction ends. Lane 1, GeneRuler™ 1 kb DNA Ladder, Lane 2, PCR reaction with pAM*katE*72, Lane 3, negative control.

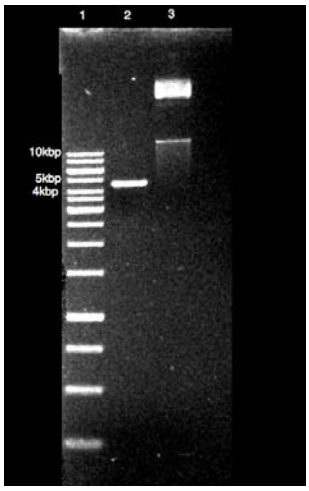

FIG. 2: *Kpn*I digestion of pBAD24. Lane 1, GeneRuler™ 1 kb DNA Ladder, Lane 2, *Kpn*I digested pBAD24, Lane 3, undigested pBAD24.

The sequence of the pBAD24R primer was: 5'-GACC-GCTT-CTGC-GTTC-TGAT-3'. Instead of DNA, groups of 6 colonies were chosen. The PCR was performed using the Biometra T-gradient thermocycler programmed to a 95ºC initial denaturation for 2 minutes, 40 cycles of 95ºC for 30 seconds, 62.6ºC for 30 seconds, and 72ºC for 2.5 minutes, and a final elongation at 72ºC for 5 minutes. Positive control included *katE*-F+*katE*-R primers and 1µl of pAM*katE*72 DNA. In the second PCR, two groups of colonies from the first PCR were selected to run in gradient PCR conditions (group 2, group 4). The PCR conditions used were the same as above except for the following: *katE* forward and reverse primers (sequence above) and a pBAD24 reverse primer were used in different combinations. The annealing temperature was a gradient of 52°C, 55.3°C, 57°C, 60°C, or 64ºC.

RESULTS

***katE* PCR amplification**. Amplification of a 2.5 kbp fragment was observed upon agarose gel visualization (Lane 2, Fig. 1). The 2.5 kbp band corresponds to the expected size of the *katE* gene, so we can be confident that we obtained our expected *katE* product. Faint non-specific high molecular weight bands at roughly 4.5 kbp and 5.5 kbp were also observed in lane 2. DNA fragments less than 250 bp were present in lanes 2 and 3. Since these fragments are also present in the negative control, they are likely produced by multimers of our primers since this is the only DNA in the negative control. Nonetheless, we can be confident we obtained our expected *katE* amplicon.

***Kpn*I digestion of pBAD24**. Comparing the digested and undigested products, there is an obvious shift in band size (Fig 2). Upon single cut digestion with *Kpn*I, a band at the expected size (4.5 kbp) was observed (Fig. 2). In the undigested sample, the plasmid migrated slower than the 10 kbp linear ladder band. This is expected since open circular DNA migrates slower than linear DNA (10). The two bands observed in lane 3 likely represent two different circular forms of the pBAD24 plasmid.

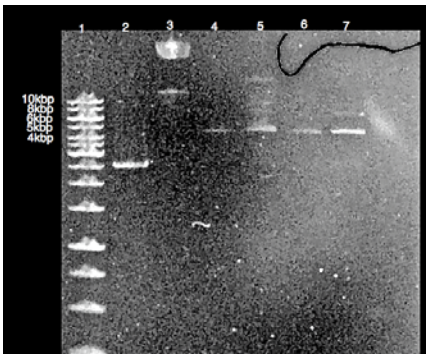

FIG 3. *Sal*I digestion of the ligation reaction to determine whether *katE* was inserted into pBAD24. GeneRuler™ 1 kb DNA Ladder, Lane 2, PCR amplified *katE*, Lane 3, undigested pBAD24, Lane 4, *Kpn*I digested pBAD24, Lane 5, ligation mixture undigested, Lane 6, *Sal*I digestion of ligation mixture using React 0, Lane 7, *Sal*I digestion of ligation mixture using React 10 buffer.

***Sal*I digestion of ligated products.** Following ligation of *Kpn*I-digested *katE* and pBAD24, confirmation of proper ligation construct was needed. To verify the presence of construct using a linear DNA ladder, ligation products were digested with *Sal*I. As *Sal*I cuts our expected pBAD24+*katE* construct one time, digestion would yield a 7 kbp product (4.5 kpb pBAD24 + 2.5 kpb *katE*). As expected, a faint 7 kbp band was observed in lane 7 following digestion (Fig. 3). This result is indicative of a successful, but inefficient ligation of our *katE* insert with pBAD24. The fainter 9 kbp fragment in the same lane suggests either multiple insertion of *katE* insert DNA into pBAD24 (2.5kb, 2.5kbp and 4.5kbp) or an incomplete digestion of multimers of pBAD24 (4.5kbp + 4.5kbp). Lanes 3 and 4 (Fig. 3) containing undigested and *Sal*I-digested pBAD24 plasmid, respectively, shows that digestion of pBAD24 occurred and yielded a 4.5 kbp linear fragment.

Colony PCR screening. We failed to detect a positive transformant containing a functional *katE* through the use of catalase-test screening (data not shown). We suspected that positive transformants did exist, but that *katE*-induction was not occurring due to inadequate diffusion of arabinose through our agar plates into the cells. Therefore, we decided to utilize colony PCR to screen for positive transformants. Since we used *Kpn*I cut sites on both ends of our *katE*-insert, both inverse and proper orientations of our insert were equally likely. To identify constructs containing *katE* in the proper orientation required for *katE* expression, the primer-pair used for colony PCR included: a reverse

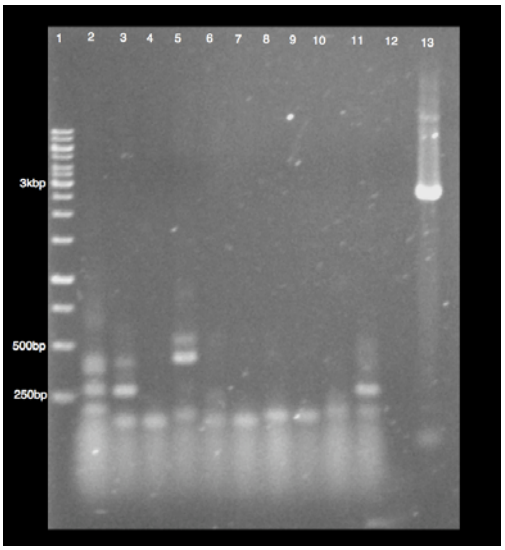

FIG. 4. Colony PCR of UM2 cells electroporated with ligation reaction. Lane 1. GeneRuler 1 kb ladder (Fermentas). Lane 2. Group 1 colonies Lane 3. Group 2 colonies, Lane 4. Group 3, Lane 5. Group 4, Lane 6. Group 5, Lane 7. Group 6, Lane 8. Group 7, Lane 9. Group 8, Lane 10. Group 9, Lane 11. Group 10, Lane 12. Negative control, no DNA, Lane 13. Positive control using pAM*katE*72 and *katE*-R and *katE*-F primers.

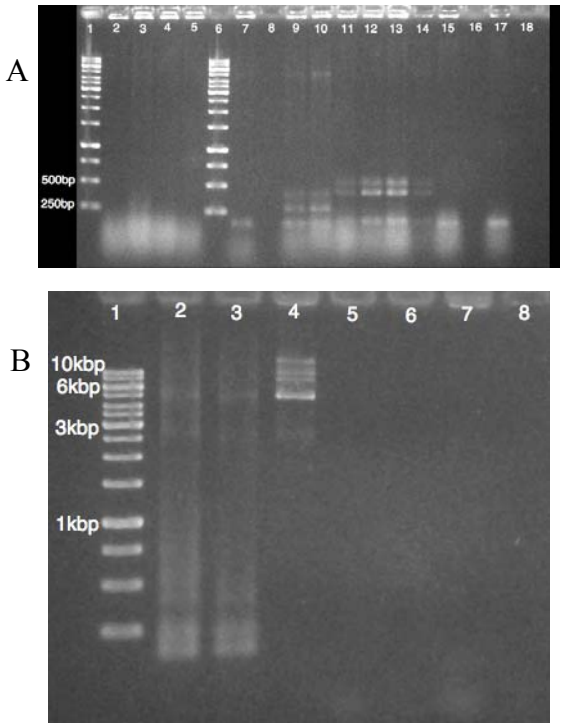

FIG 5. Agarose gel of gradient temperature PCR of UM2 colonies suspected of containing desired pBAD24 + *katE* plasmid. (A) Lane 1, GeneRuler™ 1 kb DNA Ladder, Lane 2-5 contained "group 2 colonies" using pBAD24R and *katE*-R primers, at annealing temperatures 52.1°C, 57.3°C, 60.1°C, 64°C, respectively. Lane 6. GeneRuler™ 1 kb DNA Ladder, Lanes 7, 9, and 10 contained "group 2 colonies" using pBAD24R and *katE*-F primers at annealing temperatures 52.1°C, 57.3°C, 60.1°C, 64°C, respectively. Lanes 11-14 contained "Group 4 colonies" using PBAD24R primer and *katE*-F primer using annealing temperature 52.1°C, 57.3°C, 60.1°C, 64°C, respectively. Lanes 15 and 17 contained "group 4 colonies" with pBAD24R and *katE*-R primers using temperatures 52.1°C and 60.1°C. Lane 8, 16 and Lane 18 had volumes less than 5µl and were not loaded. (B) Lane 1. GeneRuler™ 1 kb DNA Ladder, Lane 2. PCR of ligation reaction mixture using pBAD24R primer and *katE*-F primers, Lane 3. PCR of ligation reaction using pBAD24R and *katE*-F primers, Lane 4. Ligation reaction mix, Lane 5.H_20 using pBAD24R primer and *katE*-F primer, Lane 6. H_20 using pBAD24R and *katE*-R primers, Lane 7. UM2 host using pBAD24R and *katE*-F primers, Lane 8. UM2 host using PBAD24R and *katE*-R primers. Gel B, Lane 2-8 were run at annealing temperature of 55.3°C.

primer (pBAD24R) with sequence complementary to a region 83 bp downstream of the *Kpn*I recognition site on pBAD24 and a forward primer (KatE-F) that recognizes the 5' region (upstream) of *katE* template strand. Each colony PCR reaction tube contained a colony set of 6 different clones. While none of the 60 colonies tested showed an insert containing the properly oriented *katE*, an unusual banding pattern was observed in some reactions, especially for the clones represented in colony set #2 and #4 (Fig 4).

These two colony sets were further investigated because the banding pattern did not rule out the possibility that the clones possessed pBAD24 containing our *katE* insert. If the PCR amplification conditions were inadequate for amplification of the desired product, some positive transformants could be false-negatives (Fig. 4). We attempted to optimize amplification conditions by using a gradient PCR to identify false-negatives. If improper amplification was due to the annealing temperature, higher temperatures would remove the non-specific bands. In addition, the constructs from some clones could contain *katE* inserted in the reverse orientation. To identify the presence of this construct in our clones, a different primer-pair was used. Specifically, the primer-pair consisted of the aforementioned pBAD24R primer and our KatE-R reverse primer that recognizes the 3' region (upstream) of the *katE* template strand. The non-specific bands that seen in Fig. 4 (lanes 3 and 5) were still present in Fig. 5 (lanes 7-14) when the KatE-F and pBAD24R primers were used. However, none of these bands were present when the 2nd primer set (KatE-R and pBAD24R) were used (lanes 2-5, 15, 17, Fig. 5).

Endonuclease digestion of plasmid DNA isolated from transformed cells. To confirm our colony PCR results, we performed *Kpn*I digestion reactions on isolated plasmid DNA from the corresponding transformants used in the colony PCR screening.

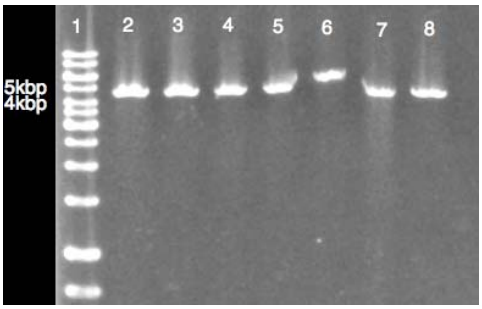

FIG 6. *Kpn*I digestion of plasmids isolated from colonies suspected of having desired *katE* + pBAD24 construct. Lane 1, GeneRuler™ 1 kb DNA Ladder, Lanes 2-7 contained digestions of plasmid DNA from colonies 15-20, respectively. Lane 8. *Kpn*I digest of pBAD24.

Consistent with our colony PCR results, *katE* insert was absent from the transformants and the expected 2.5 kbp fragment was not observed (Fig. 6). Two colonies appear to contain smaller bands that are similar in size to the bands in our colony PCR (i.e. larger than 250 bp fragments). For example, the small band observed in Fig. 6 (lane 7) is seen in Fig. 4 (lane 3). It is possible that multimers of primers were ligated into pBAD24 and this recombinant plasmid was transformed into our UM2 cells.

DISCUSSION

The aim of this study was to construct a UM2 strain complemented with a *katE* gene whose expression could be regulated in order to explain how expression affects UVA-mediated killing of catalase-deficient *E. coli*. Despite the multiple attempts made to isolate and identify a positive UM2 transformant containing a proper *katE*-pBAD24 expression construct, we were unable to do so. A systematic analysis of our findings was conducted to elucidate the potential step(s) in our cloning protocol that may have hindered our success and enable a more robust method of cloning *katE* to be developed for future studies.

Although PCR generated the desired fragment size of 2.5 kbp, a low molecular weight product that is less than 250 bp appears to have been concurrently generated in both the negative control and our experimental-condition PCR reactions. Thus, we hypothesize that these bands represent PCR products that have manifested themselves as a result of potentially non-specific primer-based interactions. In addition, these artifacts appeared to have persistently formed over a range of annealing temperatures (57^{o}C-67^{o}C) during gradient-PCR (*data not shown*). Since we did not gel purify, any of the artifacts generated in this step were subsequently carried to the rest of our experiments. Consequently, the likelihood of obtaining the desired construct is already reduced by the presence of these small fragments.

Problems during digestion and ligation could have influenced our cloning success. Figure 3 shows that our digestion conditions were sufficient for proper *Kpn*I function: the undigested circular pBAD24 vector (lane 3) became linearized upon digestion and generated the appropriately size 4.5 kbp fragment (lane 4) that is expected of pBAD24 (8). Given the inadequate resolution of our gel, we cannot differentiate the digested *katE* product from an undigested *katE* product. However, the presence of a 7 kbp fragment in lane 7, which contains *Sal*I digested ligation reaction products, indirectly suggests that our *katE* amplicon was properly digested and ligated into pBAD24. The 7 kbp fragment represents the appropriate size for a linearized construct containing the 2.5 kbp *katE* insert and 4.5 kbp pBAD24 vector. Moreover, the proposed identity of this 7 kpb band as the appropriate construct is further supported by the existence of the 2.5 kbp bands seen in lane 2 and lane 3 of Figure 5. Such bands could have been generated only if the pBAD24 containing *katE* construct was present in the ligation reaction to serve as the template due to the specific primer pairs we used (one specific for our *katE* insert and one specific for region downstream of the *Kpn*I cut site on pBAD24).

Given that our data suggests that the pBAD24 containing *katE* contruct was generated *in vitro* and that our cells were competent, our inability to isolate and identify a positive clone is likely the result of either outnumbering by competing plasmids in the ligation mix or events occurring post-transformation. For the former, the combination of pBAD24 and the aforementioned low molecular weight PCR products may have simply reduced the frequency of transformants carrying our desired construct. For the later, we propose three potential post-transformation events or host-construct incompatibilities that could lead to construct instability.

The first explanation pertains to the phenomenon known as metabolic load, which results from the over-expression of gratuitous recombinant proteins, proteins expressed that do not serve to give the host a metabolic advantage for growth under the current conditions, in *E. coli* (2, 5, 11). It has been shown that over-expression of proteins encoded in recombinant vectors leads to the disproportionate usage of cellular resources, like tRNA and rRNA, in protein production so that the cells expend a large proportion of their resources expressing vector-encoded genes rather than genes necessary for cell maintenance, growth, and division. This ultimately leads to pseudo-starvation and induction of stress

responses (degradation of rRNA), growth inhibition, and global shifts in metabolism, which collectively cause the cell to either die from the accumulation of toxic waste products as a result of metabolic shifts or enter a viable-but-non-culturable state (2, 11, 15, 16). Therefore, because *katE* serves no useful metabolic advantage to UM2 cells in non-UV conditions, we believe that plating our transformants on media containing arabinose could have led to a strong induction of *katE* expression and subsequent cell death or entrance of the bacteria into a viable-but-non-culturable state. This would explain both the lack of growth of colonies transformed with the proper construct and also why our colony screening efforts yielded no positive results.

The second potential reason for not obtaining a positive clone is due to the fact that our forward primer contains an element that is known as the Chi sequence (5'-GCTGGTGG-3'). Chi sequence containing plasmids are capable of recombining via the RecBCD pathway (17). Consequently, recombination may have lead to the loss of plasmid sequences (17).

Our third potential explanation pertains to restriction and modification systems, especially the *Eco*KI restriction and methylation system. This system serves to protect the cell from foreign DNA by recognizing non-methylated versions of a specific target sequence (5'-AACN_6GTGC-3') and cleaving the fragment. Despite not finding the exact sequence in our *katE* insert, there are numerous instances along the insert where we find a sequence that matches the *Eco*KI recognition sequence in all but one base-pair. However, because we did not use a proof-reading PCR polymerase it is extremely likely that over 30-35 cycles some of these sequences were converted into the target sequence by the introduction of a base-pair mutation (3). Since *katE* was amplified by PCR, the gene was also unmethylated or unprotected from restriction. Furthermore, our UM2 transformation host does not contain *hsdS*$^-$ or *hsdR*$^-$ mutations. HsdS is the *Eco*KI subunit responsible for target sequence recognition (14). HsdR is the *Eco*KI subunit responsible for restriction of DNA (14). We believe that it is probable that our proper construct could have been transformed into a UM2 cell, recognized by the UM2 *Eco*KI system and subsequently degraded leading to loss of amplicillin resistance conferred by the plasmid and no growth on our media containing ampicillin.

Given that our cloning efforts appeared to be successful up until transformation, these three mechanisms are potential explanations for our failure to obtain a transformant containing our construct. Still, the actual identity of the plasmids inside the transformed cells is complex. Figure 4 shows us that the colony PCR reactions failed to give us a desired 2.6 kbp product, which would only be generated from a clone that was transformed with the proper construct. However, the gel did give rise to some interesting and unusual band patterns, especially in lane 2 and lane 4. Furthermore, these band patterns were unique to only the colony sets found in lane 2 and lane 4 of Figure 4. In addition, these band patterns still remained even after gradient PCR testing (lanes 7, 9-14 in Figure 5a) while they did not appear for either of the negative controls (lanes 5 and 6 in Figure 5b) nor did they appear in the PCR-reaction that used the KatE-R and pBAD24-R primer set (lanes 2-5 and lanes 15 and 17 in Figure 5a). With all of these findings, we are led to conclude that the bands are the result of PCR amplification from something that is plasmid based. In other words, these bands represent amplification from pBAD24 plasmids that are neither re-ligated nor contain proper *katE* insert, but instead contain the low MW primer-based fragment generated during *katE* amplification (Figure 1, lane 2) in either a single-insert or multimeric-insert form. These findings that only colonies without *katE* (pBAD24 containing the low MW insert) or colonies without any insert (colonies containing empty pBAD24 vector) grew are in agreement with the results that would be expected if the aforementioned metabolic load phenomenon was occurring: the lack of any insert or the lack of an insert containing a coding region for any protein means that no gene product could be expressed and no metabolic stress could occur. In addition, this explanation is further supported by the low molecular weight band patterns specific to lanes 3 and 7 in Figure 5 that are of the same size as those seen in Figure 1, lane 2, and thus suggests that these low molecular weight fragments are in fact ligated into pBAD24. However, multimeric insertion and amplification can only account for the unique lower MW bands seen in lane 2 and lane 4 of Figure 4, and thus we propose that the remaining higher MW bands represent PCR artifacts due to non-specific interaction between our PCR primers and the plasmid. In fact, upon performing a BLAST search of the pBAD24-R primer against the pBAD24 vector sequence, we found six downstream sequences that share >7bp homology, and thus, interaction with these regions could result in the non-specific amplification necessary to generate the high MW artifacts seen in these two lanes.

The results of our study show that outnumbering by other plasmids generated in the ligation and potentially host-construct incompatibilities were responsible for our failure to obtain a UM2 strain complemented with *katE*.

FUTURE DIRECTIONS

Given the potential reasons that have inhibited our ability to isolate a proper *katE*-containing expression construct, we propose that the methods used in this experiment be optimized so the intended question of our experiment can be addressed. This optimization can be done in many ways. Firstly, the endonuclease cut sites for each primer in the primer pair should be different from one another to allow for directional cloning, which would ensure that *katE* is ligated in the proper orientation inside pBAD24. Secondly, the PCR-derived *katE* amplicon should be isolated using a gel prep and subsequently used for the remaining digestion and ligation steps so that the possibility of ligating low MW artifacts into pBAD24 is minimized. Thirdly, in order to ensure that EcoK-mediated degradation of the proper expression construct doesn't occur, we propose that an intermediate cloning-optimized cell line (i.e. DH5α) be used so that the *katE* insert can be methylated properly. Then, this properly methylated construct would be isolated via plasmid prep and transformed into UM2. Lastly, all transformants should be plated on media that does not contain arabinose so that induction of *katE* induction does not occur in order to minimze issues pertaining to metabolic load. Furthermore, the addition of glucose to the media would also help in this regard as it acts as a negative regulator for the pBAD24 promoter (8). Ultimately, these suggestions would help ensure that a positive transformant is isolated and identified, and thus allow one to begin to address how *katE* expression levels affect the UVA-mediated killing of catalase-deficient *E.Coli* strains.

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