Journal of Experimental Microbiology and Immunology (JEMI) Vol. 14: 127-134


# Antisense mRNA Method as an Alternative to Generate a Catalase Double Knockout Phenotype in a *Escherichia coli katG* Mutant

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**The purpose of this study was to generate an antisense mRNA that can conditionally mimic a catalase knockout phenotype in *Escherichia coli*. Previous studies revealed that rescue complementation of *katE*, one of the two catalase encoding genes in *E. coli*, in the catalase mutant UM2 (*kat*E2, *kat*G15) yielded an unexpected decrease in cell viability. The antisense mRNA method was to provide an alternative approach to control KatE expression by first simulating a double knockout comparable to *E. coli* UM2. By expressing a *katE* antisense mRNA under an inducible promoter in the single *katG* mutant *E. coli* strain, UM197, the double catalase knockout phenotype can be simulated in the presence of the arabinose inducer, and be rescued upon removal of the inducer. A partial sequence of the endogenous *katE* gene was amplified from *E. coli* $\chi^{760}$ wildtype strain and cloned into pBAD24 vector in an antisense arrangement. The construction and transformation of pBAD24-a*katE* into UM197 was verified by PCR amplification and was functionally assessed by comparing the relative catalase activity. It was observed that the induced UM197 with the pBAD24 control showed one-fifth the level of catalase activity compared to untransformed UM197 cells in the same media conditions, despite the absence of antisense *katE* mRNA induction. This suggested that the simulated double knockout might not have been caused solely by the antisense *katE* mRNA but may also be attributed to effects of the pBAD24 vector. However, consistent results in two catalase assays showed no catalase activity for the induced UM197 pBAD24-a*katE*. This demonstrated that the pBAD24-a*katE* construct successfully mimicked a KatE$^-$ KatG$^-$ double knockout phenotype in the *katG* knockout UM197 strain which was comparable to the UM2 double knockout positive control strain.**

The antisense RNA (aRNA) is a short (<500 nucleotides), diffusible, non-coding transcript that pairs to a target RNA at a specific complementary region of the transcript to regulate gene expression (6, 17). Naturally occurring aRNAs are important regulators of gene expression in eukaryotic and bacterial cells (chromosome-, phage-, and plasmid-encoded aRNAs) at the levels of translation, mRNA stability, and transcription (3, 7, 11, 16, 17). The accumulation of individual studies have shown that aRNAs have widespread regulatory functions in multiple bacterial species (7) and many antisense transcripts (most with unknown functions) have been identified through bioinformatics-aided searches in the *Escherichia coli* chromosome (7, 11). Known aRNA examples in *E. coli* include the DsrA RNA involved in RpoS stress response (14) and the mRNA interfering complementary RNA (micRNA) involved in OmpF/OmpC osmoregulation (15).

Antisense agents are useful for functional studies in bacteria as they can down-regulate expression of specific genes without disrupting the genome, unlike conventional knockout (KO) methods (8). Antisense RNA technology has been successfully used to manipulate gene expression in bacteria (16, 17, 18). In *E. coli*, several studies have shown successful implementation of using aRNA as a KO alternative or down regulator of important regulatory genes (3, 11-13, 18). In addition to simulating a KO or knockdown effect, aRNA expression in plasmid systems can be controlled by expressing it under either a conditional (inducible) or constitutive promoter, and by the rate of aRNA expression. Sometimes, more than one gene product is required to produce a phenotype and the loss of both genes is necessary to study the function. Double KO strains, although highly useful, are difficult to design with suitable effectiveness and viability. Antisense RNA can provide a plausible alternative to conventional double KO methods, even for essential genes required for survival.

In investigating catalase activity in *E. coli* against cell death caused by reactive oxygen species (ROS),

Cheng *et al.* (4) observed that the UM2 double KO *E. coli* strain for *katE* and *katG*, coding for the *E. coli* catalase enzymes HPII and HPI, respectively, revealed a surprising phenotype. When the UM2 double KO strain was complemented with the gene for *katE*, instead of an expected increase in cell viability, a significant decrease in cell viability was observed (4). The authors suggested that this observation may have been a result of an over-expression of *katE*, and the ensuing over-expression of catalase may be detrimental to cell viability as the strain would be oversensitive to ROS exposure.

In an effort to remedy the unexpected phenotype when rescuing with complementation, we employed the antisense mRNA method (targeting the endogenous *katE* mRNA) to provide an alternative approach in rescuing the KatE$^-$ phenotype without the problem of over expressing the catalase. Under an inducible promoter, the KO phenotype can be simulated with the addition of the arabinose inducer and be rescued upon the removal of the inducer. We report the generation of a *katE/katG* double KO from UM197 (*katG* KO) with a *katE* antisense construct, and our assessment of this aRNA construct to functionally simulate the double KO phenotype using catalase activity assays. The results of these experiments demonstrated that we successfully constructed a catalase double knock out phenotype with a *katG* single knockout using antisense *katE*.

## METHODS AND MATERIALS

**Strains used and growth conditions.** All *E. coli* K12 strains were obtained from the MICB421 Culture Collection (Table 1), with the exception of the CKLW series of cells generated in this study.

All growth was carried out at 37˚C; broth cultures were grown with shaking. General and uninduced growth was carried out in Luria-Bertani (LB) broth (1% w/v tryptone, 0.5% yeast extract, and 0.5% w/v NaCl, pH 7). For cells transformed with pBAD24, ampicillin was added to a final concentration of 100 ug/ml. For induction growth, LB broth was supplemented with L-arabinose to a final concentration of 0.2% w/v.

**Generation of competent cells.** 0.5 ml of overnight DH5α, $\chi^{760}$ and UM197 cells were inoculated into 50 ml LB broth each and grown in a shaking 37˚C water bath until an $OD_{600}$ between 0.35 and 0.45 was achieved. The cells were pelleted at 4˚C and 4000 rpm for 10 min in a Du Pont Instruments Sorval$^{TM}$ SS-34 rotor, resuspended in 25 ml of 50 mM $CaCl_2$, incubated on ice for 20 min, and centrifuged again for 10 min at 4000 rpm. The pellet was then resuspended in 5 ml of 50 mM $CaCl_2$ and incubated on ice for 20 min before 166 ul aliquot of each cell suspension was mixed with 34 ul of sterile 100% glycerol and frozen at -80˚C until use.

**Plasmid characteristic and isolation.** DH5α cells containing pBAD24 plasmid were provided by Shaan Gellatly (the University of British Columbia), and were originally obtained from the American Type Culture Collection (ATCC). The plasmid contains *araC* and *bla* promoters for arabinose-induced expression of inserts and *ampR* expression for ampicillin resistance, respectively. Cells were grown overnight and mini-prepped using GeneJET$^{TM}$ Plasmid Miniprep Kit (Fermentas, K0502) according to the manufacturer's instructions.

**TABLE 1.** Summary of *E. coli* strains used

| *E. coli* K12 strains | Genotype |
|---|---|
| DH5α | F-; *deoR; endA1; gyrA96; hsdR17(rk- mk+); recA1; relA1; supE44; thi-1;* Δ*(lacZYA-argFV169); f80lacZ; DM15* |
| MG1655 | Wild type for both *katE* and *katG* |
| UM2 | *katE2*; *katG15* |
| UM197 | *katG17::Tn10* |
| $\chi^{760}$ | Wild type for both *katE* and *katG;* parental strain for UM2 and UM197 |
| CKLW09W1 | DH5α pBAD24-a*katE** |
| CKLW09W2 | UM197 pBAD24-a*katE** |
| CKLW09W3 | UM197 pBAD24 |

*denotes antisense *katE* insert.

**PCR primers selection.** Primers for *katE* and pBAD24 vector, summarized in Table 2, were chosen using Primer3 version 0.4.0 (http://frodo.wi.mit.edu/primer3/) with forward and reverse primers being selected from a *katE* template sequence on MG1655 obtained from EcoCyc (www.ecocyc.org). A 600 bp sequence was selected with 50 bp upstream of the ribosome binding site (RBS) for *katE* (103 bp upstream of the first ATG codon). Restriction endonuclease sequences were added to the *katE* primers to ensure that the insert would be ligated in a reversed fashion to generate an antisense transcript during cloning. The pBAD24 reverse primer was selected from 300 bp of template sequence surrounding the multiple cloning site with the aid of LabLife vector database (https://www.lablife.org/g?a=vdb).

**Insert and colony PCRs and purification.** PCR thermocycling was carried out in a Biometra TGradient$^{TM}$ thermocycler with the following protocol: lid temperature at 105˚C; 95˚C (initial denaturation) for 3 min, and then 30 cycles of 95˚C (denaturation), 56˚C (annealing), and 72˚C (extension) for 1 min at each temperature, followed by a final extension of 72˚C for 10 min.

For amplification of *katE* antisense inserts, a PCR reaction was set up with 5 ul of 10x *Taq* buffer with $(NH_4)_2SO_4$ (Fermentas, B33), 4 ul of 25 mM $MgCl_2$, 1 ul of 10 mM dNTPs, 2 ul of overnight MG1655 culture, 2.5 ul of DMSO, 0.4 ul of *Taq* polymerase (Fermentas, EP0402), 33.1 ul of $dH_2O$, and 1 ul of each *katE* forward and reverse primers.

Each colony PCR reaction used to confirm presence of antisense *katE* in pBAD24 plasmid contained half the reagent volumes described for *katE* antisense PCR, with *katE* forward primer replaced with pBAD24 reverse primer. Instead of using overnight broth culture as template, a small amount of plated colony material was transferred into the reaction mix with a sterile pipette tip.

5 ul of PCR reactions were mixed with 1 ul of 6x loading dye (Fermentas, R0611) and loaded onto a 1% (w/v) agarose gel for electrophoresis. Electrophoresis was carried out at 7 V/cm for approximately 1 hr in 1X TAE buffer (50 mM Tris acetate, 2 mM EDTA, pH 8.5). Gels were then stained in a 0.5 ug/ml ethidium bromide bath prior to visualization on the Alpha Innotech AlphaImager. DNA purification was carried out using a PureLink$^{TM}$ PCR purification kit (Invitrogen, K3100-01).

**Antisense *katE* cloning.** Isolated pBAD24 and a*katE* insert restriction digestion reactions were set up as followed: 40 ul of plasmid or purified PCR inserts, 1 ul *Eco*RI (Invitrogen, 15202-013), 1 ul *Sal*I (Gibco BRL, 5217SA), 5 ul 10X Buffer O (Fermentas, BO5), and 3 ul $dH_2O$. Reaction mixes were incubated at 37˚C for 3 hr followed by inactivation at 85˚C for 20 min.

**TABLE 2.** Primer sequences (and features) used in the construction of the antisense sequences

| Primer | Restriction overhang | Sequence (5' to 3')* |
|---|---|---|
| Anti-katE-f-09w (forward *katE*) | *Sal*I | *ata*gtcgacAGTCTCCGAAGCGGGATCTG |
| Anti-katE-r-09w (reverse *katE*) | *Eco*RI | *ggg*aattcTGGGGTGATTTTGTTCGGATCT |
| pBAD4-r-09w (pBAD24 reverse) | N/A | GACCGCTTCTGCGTTCTGAT |

*Upper case nucleotides are complementary to sequence; lower case nucleotides are restriction enzyme sequence; italicized nucleotides are additional nucleotides added to complete restriction enzymes to PCR products.

*katE* insert restriction digests were purified with PureLink™ PCR purification kit (Invitrogen, K3100-01) according to the manufacturer's instructions. 1 ul of Antarctic phosphatase (New England Biolabs, M0289S) and 6 ul of 10X Antarctic phosphatase buffer (New England Biolabs, B0289S) was added to pBAD24 restriction digests, and incubated at 37˚C for 30 min and inactivated at 65˚C for 10 min.

Ligation reactions were set up using 2 ul of dephosphorylated pBAD24 vector, 4 ul digested and purified a*katE* insert, 1 ul T4 DNA Ligase (Fermentas, EL0018), 1 ul T4 DNA ligase buffer (Fermentas, B69), and 2 ul $dH_2O$. Reactions were incubated at room temperature for 1.5 hr.

**Transformation of competent cells.** 5 ul of ligation products were added to 80 ul of DH5α cells and incubated on ice for 45 min. Heat shock was carried out at 42˚C for 1.5 min. 1 ml of LB broth was added immediately after, and culture was incubated in a 37˚C shaker for 1 hr to recover. 100 ul of the cells were then plated onto LB agar plates containing 100 ug/ml ampicillin, and incubated at 37˚C overnight. Colony PCR was carried out to confirm transformation with pBAD24-a*katE*.

Positive colonies were used to inocculate overnight cultures in LB broth containing 100 ug/ml ampicillin. Overnight cultures were miniprepped using a GeneJET™ Plasmid Miniprep Kit (Fermentas, K0502). UM197 cells were then transformed with pBAD24-a*katE* and empty pBAD24 as control using the protocol for DH5α cells.

**Catalase floating disk and direct assays**. Serial dilutions of induced and uninduced $\chi^{760}$ overnight cultures were made to create a standard curve. Induced and uninduced cultures were measured at $OD_{600}$ to determine the least concentrated culture to which all of each respective cultures (i.e. induced) would be normalized to by dilution.

The floating disk assay was carried out as described by Hsieh *et al.* (10), with some modifications. Briefly, 5 mm disks of 3 mm filters (Whatman, 1030021) were dipped into the standard curve or normalized cultures for 5 sec and then dropped into 50 ml (approximately 3 cm of liquid height) of 1% (v/v) hydrogen peroxide ($H_2O_2$) in a 100 ml beaker. Catalase activity was determined as the inverse of the length of time taken for the disk to float to the top. Disks that sank back to the bottom or clung to sides of the beaker were discarded and additional trials were carried out. $H_2O_2$ solutions were replaced when bubbles formed at the bottom of the beaker.

The direct assay was performed on pelleted cells from the normalized induced and uninduced samples. 50 ul of 30% $H_2O_2$ was added to the cell material and observed for bubbling.

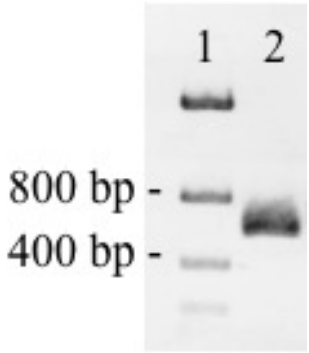


**FIG. 1. Agarose gel electrophoresis of the PCR amplification of *katE*.** Lane 1: E-Gel low range quantitative DNA ladder; lane 2: *katE* PCR product.

## RESULTS

**PCR amplification of *katE*.** To create the antisense *katE* construct, the *katE* gene was first PCR amplified from the genomic DNA of *E. coli* strain MG1655. DNA primers containing restriction endonuclease recognition sequences were used for subsequent cloning procedures. The DNA amplification was successful, showing a band size of approximately 600 bp (Fig. 1), which was the expected size of the product.

**Colony PCR of transformed *E. coli* DH5α with pBAD24-a*katE*.** In order to verify the presence of the pBAD24-a*katE* construct in the transformants, colony PCR (Anti-katE-r-09w and pBAD4-r-09w) was performed. The agarose gel showed a 600 bp DNA band in all colonies with observable differences in band intensities (Fig. 2). The amplification was shown to be distinctively robust in four of the colonies (colony #5, 7, 8 and 12) (Fig. 2).

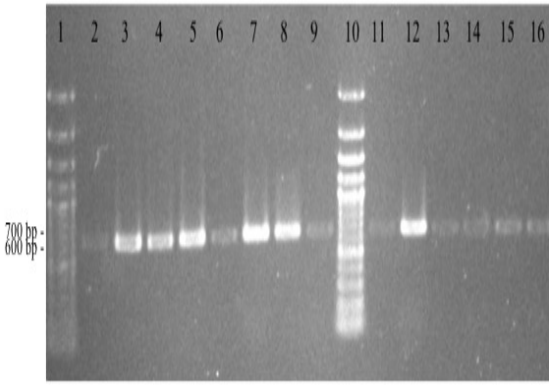


**FIG. 2. Agarose gel electrophoresis of the colony PCR of *E. coli* DH5α with pBAD24-a*katE*.** Lane 1: GeneRuler™ 100 bp Plus DNA Ladder; lane 2 to 9: colonies #1-#8, respectively; lane 10: GeneRuler™ 100 bp Plus DNA Ladder; lane 11 to 16:

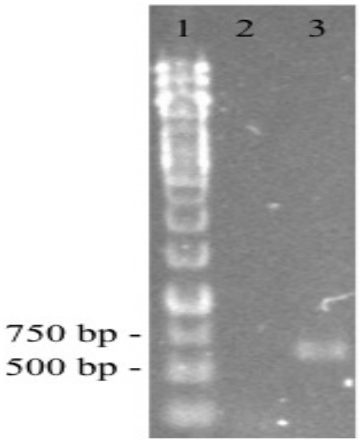


**FIG. 3. Agarose gel electrophoresis of the colony PCR of a*katE* from E. coli strain UM197 with pBAD24-a*katE*.** Lane 1: GeneRuler™ 1 kb DNA Ladder; lane 2: UM197 with pBAD24; lane 3: UM197 with pBAD24-a*katE*.

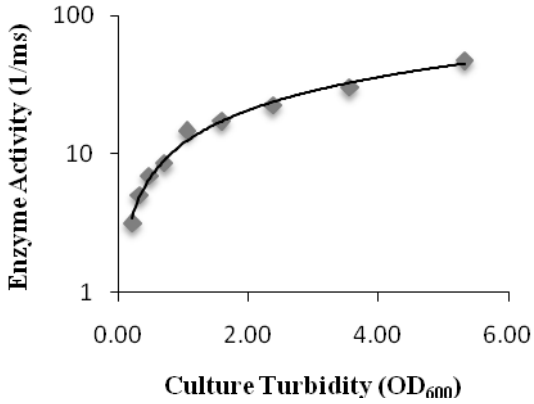


**FIG. 4. Standard curve of catalase activity filter disk flotation assay performed on arabinose-induced $\chi^{760}$ cultures.** To allow conversion of catalase activity observed in different cultures into the concentration of control cells that would be necessary to give an equivalent activity.

**Colony PCR of transformed *E. coli* UM197 with pBAD24-a*katE*.** After verification of the pBAD24-a*katE* construct, the plasmids were transformed into *E. coli* strain UM197 to create the double KO (KatE$^-$ and KatG$^-$) phenotype. As purified plasmids were used, it was expected that all transformed colonies would contain the appropriate construct; nonetheless, to verify the presence of the antisense *katE* gene, colony PCR was performed on a select colony using primer set *katE*-rv and pBAD24-rv. As expected, the agarose gel showed a 600 bp band, which corresponded to the size of the gene of interest (Fig. 3). No amplification was visible in the negative control (*E. coli* UM197 colony with pBAD24) (Fig. 3).

**Catalase activity assays on the *E. coli* bacterial cultures.** To examine the effectiveness of using the antisense mRNA strategy to knock down the catalase-positive phenotype, a catalase floating disk assay was performed by measuring the time to float as a proxy for catalase activity. Standard curves using wild type *E. coli* strain $\chi^{760}$ were constructed to allow for relative comparisons with the experimental strains (Fig. 4, Fig. 5). Both the arabinose-induced and uninduced $\chi^{760}$ cultures showed similar patterns, exhibiting exponential catalase activity (Fig. 4, Fig. 5). The functions for the induced cultures and the uninduced cultures were $y = 11.96x^{0.791}$ and $y = 10.05x^{0.871}$, respectively, where y is enzyme activity in inverse milliseconds and x is culture turbidity in $OD_{600}$.

The catalase test was performed in triplicate on each of the strains, and an average of those values was calculated. As expected, the wild type *E. coli* had the highest catalase activity, regardless of the induction condition (Table 3, Table 4). The single *katG* mutant, UM197, had slightly less catalase activity when compared to the wild type in both induction conditions (Table 3, Table 4). When induced, the UM197 strain with pBAD24-a*katE* did not demonstrate any observable catalase activity compared to its uninduced counterpart (Table 3, Table 4). In fact, the uninduced UM197 with pBAD24-a*katE* showed similar catalase activity when compared to the uninduced UM197 with pBAD24-a*katE* (Table 4). However, the induced UM197 with pBAD24 took nearly four-fold longer of

**TABLE 3.** Relative catalase activity in the floating disk assay for the controls and the strains with antisense *katE* induced by arabinose

| Strain | Enzyme Activity (1/ms) | | | Average Enzyme Activity (1/ms) |
|---|---|---|---|---|
| | Sample #1 | Sample #2 | Sample #3 | |
| $\chi^{760}$ | 29 | 29 | 31 | 30 |
| UM197 | 16 | 17 | 19 | 18 |
| UM197 pBAD24 | 5 | 5 | 4 | 4 |
| UM197 pBAD24-a*katE* | 0 | 0 | 0 | 0 |
| UM2 | 0 | 0 | 0 | 0 |

*ms=milliseconds

**TABLE 4.** Relative catalase activity in the floating disk assay for the controls and the strains with antisense *katE* without induction arabinose

| Strain | Enzyme Activity (1/ms) | | | Average Enzyme Activity (1/ms) |
|---|---|---|---|---|
| | Sample #1 | Sample #2 | Sample #3 | |
| $\chi^{760}$ | 32 | 30 | 33 | 32 |
| UM197 | 14 | 13 | 13 | 13 |
| UM197 with pBAD24 | 20 | 20 | 17 | 19 |
| UM197 with pBAD24-a*katE* | 25 | 20 | 18 | 21 |
| UM2 | 0 | 0 | 0 | 0 |

*ms = milliseconds

the time to rise compared to when it was uninduced (Table 3, Table 4). The UM197 strain without any plasmid, induced and uninduced, took a shorter time compared to its counterparts with plasmids (Table 3, Table 4).

Using the standard curve, comparisons of the relative level of catalase can be made. The time elapsed for the induced UM197 culture was equivalent to an $OD_{600}$ of 1.89 of the standardized $\chi^{760}$ (Fig. 6), which was approximately half of the equivalent turbidity of $\chi^{760}$ ($OD_{600}$ of 3.51), as expected. This value reflects that the amount of catalase in UM197 was about half of that in $\chi^{760}$ (Fig. 6). However, with the addition of pBAD24 vector, the equivalent turbidity became 0.39 when induced, which is approximately one-fifth of UM197 (Fig. 6). Surprisingly, the equivalent turbidities of the UM197 with pBAD24 cultures in different induction conditions were very different. When induced, despite the absence of the antisense *katE* mRNA, the level of catalase was only one-fifth of that when uninduced (Fig. 6).

When the pBAD24 vector contained the antisense *katE* in the induced condition, the time elapsed value was beyond extrapolation in the standard curve and therefore the equivalent turbidity reading was deemed unavailable (Fig. 6). However, in the uninduced condition, the UM197 with pBAD24-a*katE* was approximately the same equivalent turbidity as the UM197 with the empty plasmid (Fig. 6).

It should be noted that different turbidity readings were achieved on the various *E. coli* overnight cultures (Table 5). The wild type *E. coli*, $\chi^{760}$, achieved the highest OD reading in both induced and uninduced conditions (Table 5). In the UM197 cells with the vector, both with and without the antisense insert, the OD was lower by approximately 1 in the induced condition (Table 5). The overall OD values for the induced cultures were also approximately 1 OD lower than those of the uninduced (Table 5). The OD values of the uninduced cultures were roughly consistent among the various cultures, except for the lower value of UM197 (Table 5).

Further evidence that the cells, carrying antisense *katE*, had diminished catalase activity, a direct catalase assay was performed (Table 6). Consistent with the floating disk assay, no bubbling occurred for induced UM197 pBAD24-a*katE*, and induced UM197 pBAD24 showed reduced catalase activity compared to UM197 (Table 6).

## DISCUSSION

An attempt to create a double KO phenotype of the *katE* and *katG* genes by using antisense RNA encoding the *E. coli* catalase enzymes was not successful. However, a double knockout was constructed by the expression of a *katE* antisense in the *katG* mutant *E. coli* strain, UM197. In generating the antisense against the endogenous *katE* gene, primers were designed to amplify a specific 600 bp fragment from the 1.2 kb *katE* gene. In order to express the antisense mRNA, the amplified fragment was required to be ligated to the vector in an inverted position to allow expression of the opposite non-coding strand, which provided the complementary region to the *katE* transcript. By using a different restriction endonuclease enzyme on both ends, the insert orientation was controlled to ligate in the antisense fashion. Instead of amplifying the full *katE* gene, the amplified 600 bp fragment was sufficient for the antisense, which included a region complementary to the RBS in the final antisense construct. Previous studies have established that sequestration of the prokaryotic RBS using RNA antisense inhibited ribosome docking and hence translation initiation (2). Upon interaction of the *katE* antisense RNA with its endogenous target *katE* messenger transcript, it was expected that inhibition of ribosomal attachment to the transcript would inhibit its translation.

Using a floating disk catalase assay, the phenotypic consequence of the expression of the *katE* antisense

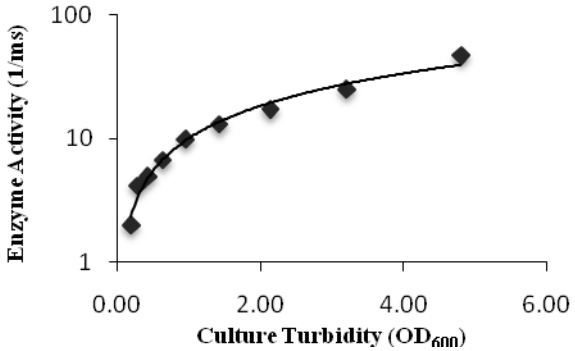


**FIG. 5. Standard curve of catalase activity floating disk assay performed on arabinose-uninduced $\chi^{760}$ cultures.** To allow conversion of catalase activity observed in different cultures into the concentration of control cells that would be necessary to give an equivalent activity.

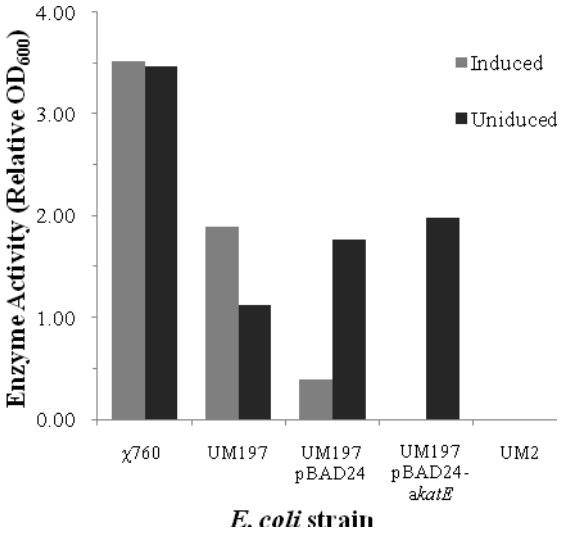


**FIG. 6. Comparison of the relative concentration of catalase activity in different strains.** Activity is expressed as the turbidity of $\chi^{760}$ strain that would give the observed catalase activity.

was confirmed. The experimental results reflected the relative catalase activity of each strain dependent on the amount of time each sample required to reach the surface of the $H_2O_2$ solution; a greater time would reflect a lower degree of catalase activity and vice versa. Interestingly, when compared with the UM197 cells transformed with the pBAD24 vector lacking the *katE* antisense in the presence of the arabinose inducer, an obvious decrease in the catalase activity occurred when compared to the same cells without the presence of arabinose (Table 3, 4). Furthermore, this phenomenon of decreased catalase activity occurred in all three trials (Table. 3, 4 and data not shown), eliminating the likeliness of experimental error. It has been speculated that the arabinose induction caused a thrust in the transcription of the high copy plasmid pBAD24 (1) to facilitate the use of arabinose through the pBAD24 *araC* gene, encoding for the AraC regulatory protein, which activated pBAD24 transcription in the presence of arabinose (5). It has been previously shown that in the presence of plasmids in high copy numbers, an insufficiency in the amount of available transcriptional machinery components can result (6). Reasonably, as pBAD24 is a high copy plasmid, its AraC activated transcription (under arabinose influence) may have resulted in an insufficient availability of transcriptional factors, other components of the transcriptional machinery and free nucleotides which are normally devoted to the transcription of the bacterial chromosomal genes, including *katE*, to the levels of non-transformed UM197 cells. This leads to the rationalization that the observation of decreased catalase activity in the presence of arabinose may be caused by a lack of adequate levels of endogenous *katE* transcription.

These results were further supported by the direct catalase assay where the degree of bubbling was analyzed when cells were exposed to 30% $H_2O_2$ as the arabinose induced pBAD24–transformed UM197 cells showed slower and fewer formation of bubbles compared to the uninduced cells and $\chi^{760}$ cells, which showed similar results in both the arabinose induced and uninduced assays (Table 6). An important issue to address is whether the sequence downstream of the *araC* promoter in the pBAD24 empty vector, upon its transcription in the presence of arabinose, affected the cell growth and transcription of chromosomally localized genes, including *katE*, in the UM197 strain. If this were the case, then the effect of the decrease in catalase activity of the negative control cells under arabinose induction may have been contributed by the transcribed product from the pBAD24 empty plasmid and/or its protein counterpart.

Despite the catalase activity–reducing effect of the pBAD24 vector, the presence of the pBAD24–*katE* antisense showed a degree of catalase activity much

**TABLE 5.** Turbidity readings of overnight *E. coli* cultures at $OD_{600}$

| Strain | Induced | Uninduced |
|---|---|---|
| $\chi^{760}$ | 4.80 | 5.33 |
| UM197 | 4.64 | 3.74 |
| UM197 pBAD24 | 3.68 | 5.41 |
| UM197 pBAD24-a*katE* | 3.53 | 4.95 |
| UM2 | 4.40 | 4.49 |

**TABLE 6.** Catalase activity assay of *E. coli* culture cell pellets compared by bubble formation after addition of 30% $H_2O_2$

| Strain | Catalase Activity* | |
|---|---|---|
| | Induced | Uninduced |
| $\chi^{760}$ | +++ | +++ |
| UM197 | ++ | ++ |
| UM197 pBAD24 | + | ++ |
| UM197 pBAD24-a*katE* | - | ++ |
| UM2 | - | - |

*- indicates no bubbles, + indicates slow and small bubbles, ++ indicates fast and small bubbles, +++ indicates fast and big bubbles.

lower than the UM197 cells induced with the empty vector alone, and similar to that of the *E. coli* UM2 (*katE*/*katG* KO strain). These findings suggested that the pBAD24-a*katE* rendered the catalase activity of the *E. coli* UM197 *katG* single KO strain with a catalase phenotype comparative to that of the UM2 double KO strain.

In analyzing the growth of the different strains of *E. coli* used in this experiment with and without the presence of arabinose, it was observed that the uninduced UM197 cells transformed with pBAD24 exhibited a higher density of cells compared to the UM197 non-transformed cells (Table 5). This might be attributed to a variation in the growth in that particular culture of cells and has been disregarded for subsequent experiments using those cells. In addition, the observation of a 1 OD decrease in the cells transformed with pBAD24 (with and without *katE* antisense insert) can be explained by a reasoning similar to the phenomenon of the decreased catalase activity in the pBAD24 transformed UM197 cells under the influence of arabinose, an insufficiency of transcriptional machinery components (6). An inadequate amount of these components would deter the growth of the bacterial cells as cell survival and growth genes would not be transcribed and expressed abundantly to support the robust growth of the $\chi^{760}$ wild type cells. The observed decrease in cellular growth in these cells could also be attributed to an increased metabolic burden as the maintenance of high copy plasmids, such as pBAD24, under induction conditions have been shown to produce a negative effect on the degree cell growth (9).

In this study, it was demonstrated that the successful application of RNA antisense technology using a pBAD24 plasmid vector to the single *katG* KO *E. coli* strain UM197 created a double $KatE^-$ $KatG^-$ KO phenotype. This double KO phenotype showed a level of catalase activity in the floating disk catalase assay that was similar to the level in the true *katE*/*katG* double KO *E. coli* UM2 strain.

## FUTURE DIRECTIONS

Ultimately, the suitability of UM197 pBAD24-a*katE* for use in replacing the complementation method in work carried out by Cheng *et al.* relies on cell viability. Viability comparisons between uninduced UM197 pBAD24-a*katE* cells should be compared with previously generated *katE* complemented UM2 cells (4). Once comparisons have been made, future functional studies of *katE* can select the most suitable strain for experiments.

This study lacked confirmation of function for the a*katE* sequence in the knockdown of catalase activity. Future efforts should repeat the floating disk test but focus on generating a UM197 strain transformed with pBAD24 with 600 bp insert unrelated to *katE* to confirm *katE* specific activity was observed in UM197 cells containing pBAD24-a*katE*.

Growth curve studies should be performed on UM197 cells containing pBAD24-a*katE*, to elucidate the growth pattern of these transformed cells. A complete growth curve analysis should confirm whether pBAD24-a*katE* will lead to abnormal cellular physiology and whether it is a suitable plasmid for knocking down activity of *katE* in functional studies. By extension, should growth effects render pBAD24 an unusable vector, alternative inducible vectors should be tested.

### ACKNOWLEDGEMENTS

We would like to thank Dr. William Ramey and Matthew Mayer for the unparallel support, technical assistance and guidance throughout the study. We would also like to thank Shaan Gellatly for providing us with the pBAD24 vector.

### REFERENCES

1. **Banerjee, S., S. Salunkhe, A. Apte-Deshpande, N. Mandi, G. Mandal, and S. Padmanabhan.** 2009. Over-expression of proteins using a modified pBAD24 vector in *E. coli* expression system. Biotechnol. Lett. **31:**1031-1036.
2. **Brantl, S.** 2002. Antisense-RNA regulation and RNA interference. BBA – Gene Struct. Expr. **1575:**15-25.
3. **Chen, G., C. L. Patten, and H. E. Schellhorn.** 2003. Controlled expression of an *rpoS* antisense RNA can inhibit RpoS function in *Escherichia coli*. Antimicrob. Agents Chemother. **47:**3485-3493.
4. **Cheng, M., A. Chow, J. Ho and B. Luk.** 2009. *katE* complementation fails to protect against UV-A-mediated killing in catalase-deficient Escherichia coli. J. Exp. Microbiol. Immunol. **13:**63-66.
5. **Cronan, J. E.** 2006. A family of arabinose-inducible *Escherichia coli* expression vectors having pBR322 copy control. Plasmid **55:**152-157.

6. **da Silva, N.A. and J. E. Bailey.** 1986. Theoretical growth yield estimates for recombinant cells. Biotechnol. Bioeng. **28:**741-746.
7. **Dornenburg, J. E., A. M. DeVita, M. J. Palumbo, and J. T. Wade.** 10 March 2010. Widespread Antisense Transcription in *Escherichia coli*. mBio. doi:10.1128/mBio.00024-10.
8. **Dryselius, R., A. Nikravesh, A. Kulyté, S. Goh, and L. Good.** 2006. Variable coordination of cotranscribed genes in *Escherichia coli* following antisense repression. BMC Microbiol. **6:**97-97.
9. **Glick, B. R.** 1995. Metabolic load and heterologous gene expression. Biotechnol. Adv. **13:**247-261.
10. **Hsieh, S., S. Hwang, K. Kim, and S. Kim.** 2004. Investigation of the potential UV-A enhanced toxicity and the potential inhibition of catalase by phthalate exposed to UV-A. J. Exp. Microbiol. Immunol. **12:**50-56.
11. **Kemmer, C. and P. Neubauer.** 2006. Antisense RNA based down-regulation of RNaseE in *E. coli*. Microb. Cell. Fact. **5:**38.
12. **Kim, J. Y. and H. J. Cha.** 2003. Down-regulation of acetate pathway through antisense strategy in *Escherichia coli*: improved foreign protein production. Biotechnol. Bioeng. **83:**841-853.
13. **Krylov, A. A., L. G. Airich, E. M. Kiseleva, N. I. Minaeva, I. V. Biryukova, and S. V. Mashko.** 2010. Conditional silencing of the *Escherichia coli pykF* gene results from artificial convergent transcription protected from rho-dependent termination. J. Mol. Microbiol. Biotechnol. **18:**1-13.
14. **Majdalani, N., C. Cunning, D. Sledjeski, T. Elliot, and S. Gottesman.** 1998. DsrA RNA regulates translation of RpoS message by an anti-antisense mechanism, independent of its action as an antisilencer of transcription. Proc. Natl. Acad. Sci. U. S. A. **95:**12462-12467.
15. **Mizuno, T., M. Y. Chou, and M. Inouye.** 1984. A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). Proc. Natl. Acad. Sci. U. S. A. **81:**1966-1970.
16. **Nellen, W. and C. Lichtenstein.** 1993. What makes an mRNA anti-sense-itive? Trends Biochem. Sci. **18:**419-423.
17. **Wagner, E. G. and R. W. Simons.** 1994. Antisense RNA control in bacteria, phages, and plasmids. Annu. Rev. Microbiol. **48:**713-742.
18. **White, D. G., K. Maneewannakul, E. von Hofe, M. Zillman, W. Eisenberg, A. K. Field, and S. B. Levy.** 1997. Inhibition of the multiple antibiotic resistance (mar) operon in Escherichia coli by antisense DNA analogs. Antimicrob. Agents Chemother. **41:**2699-2704