Journal of Experimental Microbiology and Immunology (JEMI) Vol. 18: 139 – 145

Partial Confirmation of Single *katG* and *katE* Knockouts and Double *katG/katE* Knockouts Created from Isogenic Background of *Escherichia coli* K-12 Strains

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Presumptive knockouts of *katE* and *katG* catalases were constructed from BW25113 *E.coli* K-12 strain background via Lambda Red recombination system to generate *kat*E/*kat*G double knockout for a better assessment of the roles of the individual catalases (Narita and Peng, JEMI, 16, 123-8, 2012). The kanamycin resistance cassettes were then removed through FLP-FRT recombination system for consistent antibiotic sensitivity across the laboratory strains. In this study, our goal was to confirm the genotype and phenotype of these knockout strains by PCR, and catalase activity assay with 30% or 2% hydrogen peroxide (H_2O_2). The *kat*G single knockout and double knockout strains, as expected, were catalase positive and negative, respectively. The *katE* single knockout strain was only catalase positive when the test was done with 2% hydrogen peroxide suggesting a threshold concentration of hydrogen peroxide required for *kat*G expression. The PCR results confirmed the continued existence of *kat*E knockout during the process of creating double knockouts. It also identified that the *kanR* gene insert is present in the presumptive double knockout strain PN11W-4a.

Reactive oxygen species (ROS) are known to react and disrupt many biological molecular processes that are vital for many bacteria. H_2O_2 generates lethal ROS (1,2). As a consequence, H_2O_2 activates the OxyR regulon in *Escherichia coli* (1,3). One of the genes that the OxyR protein regulates is the *katG* gene which synthesizes a catalase to counteract the harmful effects of the ROS (1). Another gene, *katE*, also produces another protective catalase; however, it is not regulated by the *OxyR* regulon. *KatE* is permanently induced under aerobic conditions and therefore acts upon H_2O_2 at a much quicker pace (1,2).

Mutant strains of *E. coli* have been used to determine the individual effects of the two catalase genes. However, there has been insufficient direct relationship between the parental and the knockout strains for evaluation, since some knockouts used in the same experiment were derived from different parental strains (4,5). Another problem is the addition of complicating factors such as transforming cells with plasmid vectors to generate or analyze the catalases in the double knockout strains (6,7). In a previous study, Narita and Peng (9) created catalase knockout strains from a single parental BW25113 *E.coli* K-12 strain background for more accurate isogenic comparisons. However, no PCR verification was done to confirm the existence of the knockouts (8,9). The knockout was performed via site specific mutagenesis using Lambda Red recombination system to insert a kanamycin resistance gene in place of the target gene. This *kanR* gene encodes an aminoglycoside phosphotransferase which is then removed via FLP-FRT recombination system. This recombination system is not without error and may leave behind a FRT sequence or a scar region in the target gene locus. In this experiment our objective was to confirm the identities of the knockouts created in the prior study by PCR verification and assessment of catalase activity and kanamycin resistance. For this purpose, different primer sets flanking regions around the *katE* and *katG* and within the aminoglycoside phosphotransferase gene (*kanR)* were used to verify a complete, scar-free deletion of *katG* and *katE* gene locus.

It is speculated from previous studies that a certain threshold of H_2O_2 is required to activate *katG* (2,10). If threshold is not reached, other genes such as *kat*E and alkyl hydroperoxide reductase (*ahpCF)* take part in breaking down H_2O_2 (2,10). Catalase tests were also performed to confirm the catalase activity in the constructed strains. Identifying the successful knockout in these strains from isogenic background, could lead to a more in-depth and thorough analysis of the catalases in question and their function in the cellular environment.

MATERIALS AND METHODS

The *katE* and *katG* single knockout strains (9) and the *katE*/*katG* double knockout strain (11) were created with kanamycin resistance cassette insertion using the Lambda Red recombination system. Narita and Peng continued to remove the antibiotic cassettes from the *katE*/*katG* double knockout and *katE* and *katG* single knockout strains using the FLP-FRT recombination system to ensure consistency in antibiotic resistance within the generated strains (9). PCR verification and catalase test were performed in this experiment to confirm the knockouts.

Bacterial strains and growth condition. The *Escherichia coli* strains (from the University of British Columbia, Department of Microbiology & Immunology, MICB 421 culture collection) used in this study are listed in Table 1. The strains were grown on agar plates (1.6% tryptone, 1% yeast extract, 0.5% NaCl, 1.5% agar) supplemented with 25 μg/ml kanamycin for kanamycin resistance assay and incubated at 37°C. Overnight cultures were prepared by inoculating 5 ml Luria broth and incubating at 37°C.

Cell washing for template. Templates to be used for PCR amplification were prepared according to the modified method used by Zhu (12). 1.5 ml of *E. coli* culture, with an OD_{600} between 0.6 and 1.0, was centrifuged at 15700 g for 5 minutes. After the supernatant was removed, the cell pellet was washed with 0.5 ml 1 M NaCl two times. Then to remove residual NaCl and to create a nuclease-free environment, the pellet was washed

with 0.5 ml Invitrogen™ diethylpyrocarbonate (DEPC) treated pyrogen-free water. The pellet was resuspended in 50 ul DEPC treated water. Using DEPC treated water, 1/100 dilutions of the templates were made and used later on for PCR.

TABLE 1. *Escherichia coli* strains used in this study.

Strain	Genotype
JW1721-1 (*katE* single knockout strain)	F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, ΔkatE731::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514
JW3914-1 (*katG* single knockout strain)	F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, rph-1, Δ(rhaD-rhaB)568, ΔkatG729::kan, hsdR514
PN11W-1 (*katE* single knockout strain without kanamycin resistance cassette)	F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, Δ*katE*731, rph-1, Δ(rhaD-rhaB)568, hsdR514
PN11W-2 (*katG* single knockout strain without kanamycin resistance cassette)	F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, rph-1, Δ(rhaD-rhaB)568, ΔkatG729, hsdR514
PN11W-3 (presumptive catalase double knockout strain with kanamycin resistance cassette)	F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, ΔkatE731, ΔkatG729::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514
PN11W-4a/b (presumptive catalase double knockout strains with kanamycin resistance cassette removed)	F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, ΔkatE731, ΔkatG729, rph-1, Δ(rhaD-rhaB)568, hsdR514

PCR amplification. The primers used are listed in Table 2 were from Integrated DNA Technologies. The K primers are complementary to sites within the kanamycin resistance gene to confirm the presence in the genome and successful knockouts. The E and G primers are complementary to sites flanking the *E. coli katE* and *katG* genes respectively to identify the presence of the loci in the genes. PCR was performed in 10 ul reactions containing 1 ul of 10X PCR buffer (Invitrogen, #Y02028), 0.8 U Taq DNA polymerase (Thermo Science, #EP0404), 1.0 uM each of forward and reverse primers, 1 mM dNTPs (Fermentas, #Y02016), 3 mM $MgCl_2$ (Invitrogen™, #Y02016) and DEPC-treated water (Invitrogen™, #46-2224). Denaturation was set at 95°C for 5 min, followed by 35 cycles at 94°C for 45 seconds, appropriate annealing temperatures for 45 seconds and 72 °C for 3 minutes, followed by final extension at 72 °C for 8 minutes. Calculated appropriate annealing temperatures were acquired using New England Biolabs Inc. online T_M Calculator and is shown on Table 3

Gel electrophoresis. PCR products were separated on 1.0% (w/v) agarose gel to observe the presence or absence of bands for *KatE* (~2.2 kb), *KatG* (~2.2 kb), *KanR* (~1.4 kb), partial *KanR* gene with peripheral base pairs (~1.7 kb for primer sets 2 and 3; ~0.7-1kb for primer sets 5 and 6), and/or a scar region (~0.4 kb). 1X Tris-acetate-EDTA (TAE) buffer was used for preparing gels and for electrophoresis. 1 ul 6X DNA Loading dye (Thermo Science, #R0611) was mixed with 5 ul PCR product. The ladders used were Thermo Science GeneRuler™ 1kb DNA Ladder (0.5 ug/ul; #SM0311), Invitrogen™ High DNA Mass Ladder, and Invitrogen™ Low DNA Mass Ladder (#10068-013). Electrophoresis was done at 120 V for 1.1 hours at room temperature. Staining was done by soaking the gel for approximately 15-20 minutes in 0.5 µg/ml ethidium bromide. Gel imaging was done with ultraviolet light using the MultiImage light cabinet (Alpha Innotech®).

Catalase activity test. Each strain was tested for the presence of catalase. A drop of 2% or 30% hydrogen peroxide was placed on top of a slide. A colony from an LB agar plate streaked and incubated at 37 °C for 18 to 24 hours beforehand was placed on the hydrogen peroxide with an inoculating loop. A positive test was indicated by vigorous bubbling and a negative test was displayed by very little or no bubbling. The test was replicated using colonies from 2 subcultures from the same source strain to confirm results.

TABLE 2. Primer sequences used to amplify *katE*, *katG*, and *kanR* genes from *E. coli* strains.

Gene	Bottom Strand primer (5' → 3')	Top strand primer (5' → 3')
kanR	CAGTCATAGCCGAATAGCCT (K1)	CGGTGCCCTGAATGAACTGC (K2)
katE	AAATTAAGGAGACGAGTTCAA (E1)	CGCAATTGCGCCGCCTC (E2)
katG	CTGTAGAGGGGAGCACATTGATG (G1)	AGCCGCTGAACGGGGTC (G2)

RESULTS

Kanamycin sensitivity. The sensitivity results of each *E. coli* strain to kanamycin are summarized in Table 4. JW1721-1, JW3914-1, and PN11W-3 strains were kanamycin resistant. PN11W-1, PN11W-2, and PN11W-4b strains were sensitive to kanamycin. PN11W-4a strain, which was expected to have the kanamycin resistance cassette removed, grew on kanamycin LB agar plates. Kanamycin resistance phenotype expressed by all the strains were of those expected except for PN11W-4a which appeared to be kanamycin resistant.

Catalase activity. Catalase activity of all the *E. coli* strains was measured to assess the phenotypes of the knockout strains (Table 4). JW3914-1 and PN11W-2 strains, which were *katG* single knockouts, were catalase positive when tested for catalase activity and with 30% and 2% H_2O_2. This was expected since the strains still have the *kat*E gene to express a catalase. Bubble formation was approximately two times more vigorous in response to 2% H_2O_2. JW1721-1 and PN11W-1 strains, which were *kat*E single knockouts, did not form bubbles in response to 30% H_2O_2 but they were observed to be catalase positive in response to 2% H_2O_2. JW1721-1 strain resulted in a milky turbidity in response to the 30% H_2O_2. These strains still had the *kat*G gene and the catalase expressed by it was expected to break down H_2O_2. PN11W-3 and PN11W-4a/b were catalase negative in response to both 30% and 2% H_2O_2. These were expected results since both *kat*E and

*kat*G genes were expected to be knocked out from these strains.

TABLE 3. Primer sets used to amplify *katE*, *katG*, and *kanR* genes from *E. coli* strains.

Primer Set Number	Primer Sets	Calc. T_M/ Exp. T_M[1]	Purpose
1	E1E2	57°C / 58°C	Observe gene presence in the *katE* site of the *katE* locus
2	E1K2	55°C / N/A	Observe *KanR* presence in the *katE* site of the *katE* locus, if present
3	E2K1	52°C / N/A	Observe *KanR* presence in the *katE* site of the *katE* locus, if present
4	G1G2	53°C / 58°C	Observe gene presence in the *katG* site of the *katG* locus
5	G1K2	53°C / N/A	Observe *KanR* presence in the *katG* site of the *katG* locus, if present
6	G2K1	52°C / >54°C	Observe *KanR* presence in the *katG* site of the *katG* locus, if present

[1]Experimental T_M was discovered in our experiments, when applicable.

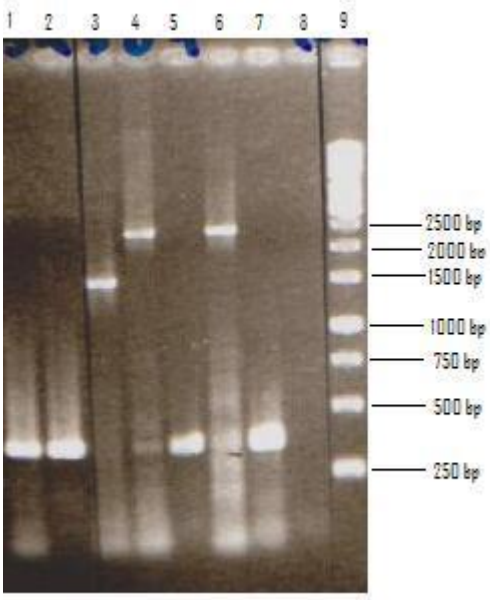

FIG. 1. Profile of the *katE* locus among 7 different strains of PCR products using primer set 1. Lanes 1 and 2 were strains PN11W-4a and PN11W-4b respectively at a PCR annealing temperatures of 56°C. Lanes 3-8 were strains JW1712-1, JW3914-1, PN11W-1, PN11W-2, PN11W-3, and PN11W-4a respectively at a PCR annealing temperature of 58°C. Lane 9 was the GeneRuler™ 1kb DNA ladder bands. Bands of the *katE* gene, *kanR* gene, and the scar region are revealed in this profile.

PCR verification and gel electrophoresis. To further verify the genotype of each strain, we performed PCR with proposed primer sets to find the presence or absence of *katG*, *katE*, and kanamycin resistance cassette genes (Tables 2 and 3). A temperature gradient PCR was also performed to find the optimal temperature for each primer set (Table 3). Using the set 1 primers (E1/E2) that flank the sides of the *katE* locus, we attempted to confirm and present the genes located within the locus. After testing multiple annealing temperatures for optimization with primer set 1, we concluded that the PCR showed the best results at 58°C (Fig. 1). PCR verification results of the *kat*E locus came out as expected (Fig. 1). Strain JW1712-1, the *katE* knocked out with the *kanR* gene, displayed the expected length of the *kanR* gene of ~1.4 kb on the gel (Fig. 1). Both JW3914-1 and PN11W-3 strains displayed the *katE* gene with the expected length of ~2.2 kb, and a ~0.4 kb band was observed for strains PN11W-1 and PN11W-3 as a result of kanamycin gene removal by FLP-FRT recombination system (12). PN11W-4a and PN11W-4b also showed the ~0.4 kb band at the right length in the gel, but due to loading error, results can be only seen in our results at a lower annealing temperature of 56°C (Fig. 1). Results from PCR done with the annealing temperature of 56°C were consistent with those at the annealing temperature of 58°C but with fainter bands (data not shown).

We had difficulties with our PCRs so were unable to present a full gel electrophoresis profile of primer sets 2 (E1/K2) and 3 (E2/K1). However, we were able to achieve some results with the JW1721-1 strain with these two sets at a PCR annealing temperature of 50°C (data not shown).

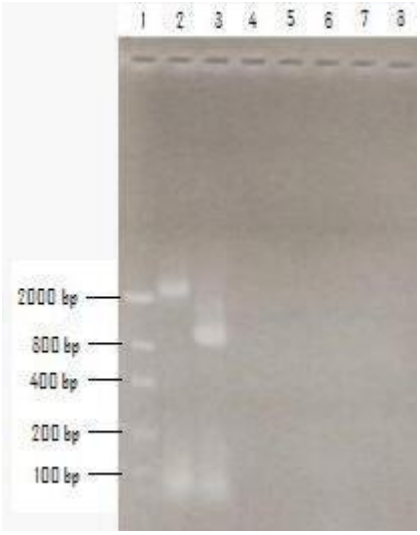

FIG. 2. Exposure edited profile of the *katG* locus among 7 different strains of PCR products using primer set 4. Lanes 2-8 were strains JW1712-1, JW3914-1, PN11W-1, PN11W-2, PN11W-3, PN11W-4a, and PN11W-4b respectively at a PCR annealing temperature of 58°C. Lane 1 was the Low DNA Mass™ Ladder bands.Bands revealing either *KanR* gene or *katG* gene were visible for strains JW1712-1 and JW3914-1.

TABLE 4. Summarized patterned results of the phenotype and partial genotype of the 7 *E. coli* strains.

Strain	Kanamycin Sensitivity	Catalase Activity (with 30% H_2O_2)[1]	Catalase Activity (with 2% H_2O_2)[1]	Detected PCR gene product with correspond primer set
JW1721-1	Resistant	- (with milky turbidity)	+	*katE* locus: *kanR* with set 1 *katG* locus: *katG* with set 4
JW3914-1	Resistant	+	++	*katE* locus: *katE* with set 1 *katG* locus: *kanR* with set 4 and set 6
PN11W-1	Sensitive	-	+	*katE* locus: Scar region with set 1
PN11W-2	Sensitive	+	++	*katE* locus: *katE* with set 1
PN11W-3	Resistant	-	-	*katE* locus: Scar region with set 1 *katG* locus: *kanR* with set 6
PN11W-4a	Resistant	-	-	*katE* locus: Scar region with set 1 *katG* locus: *kanR* with set 4
PN11W-4b	Sensitive	-	-	*katE* locus: Scar region with set 1

[1]Catalase activity was exhibited either with bubble formation (+), vigorous bubble formation (++), or little or no bubble formation (-)

PCR product gel analysis of JW1721-1 showed the expected ~1 kb bands for primer set 2 and ~0.6 kb for set 3, confirming the presence of *kanR* on the *kat*E locus.

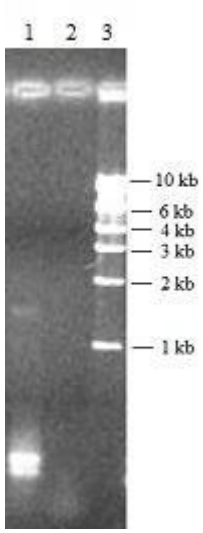

FIG. 3. Profile of the *katG* locus among 2 different strains of PCR products using primer set 4. Lanes 1 and 2 were strains PN11W-4a and PN11W-4b respectively at a PCR annealing temperature of 58°C. Lane 3 was the High DNA Mass™ Ladder bands. Bands revealing the size of *KanR* gene were visible for the PN11W-4a strain.

To generate the same sort of profile for the *katG* locus, we attempted to optimize the PCR conditions using primer set 4 (G1/G2). However, due to cell death potentially from old age and time constraints, we were only able to produce results for the parental strains instead of a full profile of all the strains with this primer set (Fig. 2). At a PCR annealing temperature of 58°C strains, JW1721-1 and JW3914-1 displayed the expected band lengths of ~2.2 kb and ~1.4 kb, respectively, representing the presence of *katG* and *kanR* gene in those strains. Using freshly cultured PN11W-4a cells, we were able to display a ~1.4 kb band with primer set 4, confirming the presence of the *kanR* gene on this strain (Fig. 3).

Primer sets 5 (G1/K2) did not generate any positive results due to PCR ratio errors caused by incorrect pipette volume measurement (data not shown). Primer set 6 encountered similar errors but was still able to produce some faint expected bands which were visible after an inversion of colour and exposure edit was done on the gel photo (Fig. 4). At a PCR annealing temperature of 55°C strains, JW3914-1 and PN11W-3 showed very faint but expected bands at around 0.7 kb (Fig. 4). These bands represented the regions located in the flanking regions of the *katG* gene and a region on the *kanR* gene in the *katG* locus. In the same figure, strain JW3914-1 also produced an extremely faint band in the same primer set at a lower PCR annealing temperature of 54°C, but the band previously produced by PN11W-3 was no longer clearly visible. Due to loading the incorrect ladder in the gel, weused an alternative way to calculate band length. The distance from the top of the wells to the faint bands on Fig. 4 was measured in cm and then divided by the distance from the top of the wells to the gel front (marked by component residuals on gel) on the same figure. This calculated ratio was then compared to the gel with the correct ladder (Fig. 2) by multiplying the ratio to the

distance from the top of the well to the gel front of the gel with the correct ladder. The resulting number was the distance measured from the top of the well to a certain point in the gel with the correct ladder. This calculation gave an approximate length of 0.7 kb.

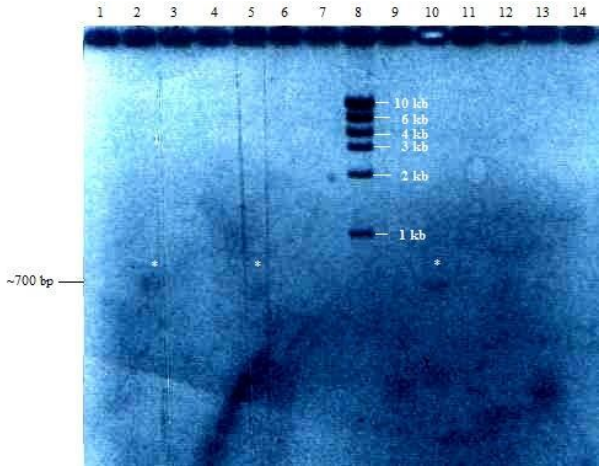

FIG. 4. Inverted colour and exposure edited profile of the *katG* locus among 7 different strains of PCR products using primer set 6. Lanes 1-7 were strains JW1712-1, JW3914-1, PN11W-1, PN11W-2, PN11W-3, PN11W-4a, and PN11W-4b respectively at a PCR annealing temperature of 55°C. Lane 8 were High DNA Mass™ Ladder bands. Lanes 9-14 has the same strain order but without PN11W-4b and done at a PCR annealing temperature of 54°C. Bands revealing the size of partial *KanR* gene and *katG* peripheral bases were faintly visible for strains W3914-1, PN11W-3 in lanes 2 and 5. Lane 10 also produced faint bands for the W3914-1 strand in lane 10. Bands marked below "*".

DISCUSSION

The aim of this study was to verify the *katE* and *katG* knockouts created in a prior study (9) from the same *E. coli* parental strain background by PCR, catalase activity, and kanamycin resistance tests. PN11W-4a growth in the presence of kanamycin was unexpected since the kanamycin cassette was supposedly removed by FLP-FRT recombination system transformed into the PN11W-3 strain (8,9). Later in the PCR results we observed the presence of the *kanR* band in the kat*G* locus.

The presence or absence of *katE* was verified successfully using the E1/E2 primer set and the results were as expected (Fig. 1). The product bands indicated background-specific amplification. Primer sets 2 and 3 were designed to identify the improper retention of the *kanR* gene in the double knockout strains from unsuccessful removal by the FLP-FRT recombination system. The temperature gradient PCR performed with primer sets 2 and 3 did not show results for sufficient evaluations, potentially due to the age of the templates, since the samples were stored frozen but in -20°C water for long periods leaving them susceptible to degradation after being prepared. Technical errors in pipetting calibrated volumes while preparing the PCR reaction tubes also contributed as a source of error. Due to limited time, we were unable to replicate and fine-tune our experiment. Yet, running those sets again is encouraged for more accurate confirmation of the continued existence of *kat*E knockout.

The specificity of amplification for primer set 4 was previously shown by Narita and Peng (9) and resulted in product bands in the negative and positive control of JW strains (Fig. 2). We were able to confirm the presence of *kanR* on the *katG* locus in the PN11W-4a strain indicating that mutagenesis in this strain was unsuccessful and it does not exhibit a double knockout genotype and phenotype (Fig. 3). However, this set failed to generate bands for other experimental PN11W strains possibly due to age of the template, speculating that the mutated strains may have died faster than the parental strains as genetic alteration might lower their survivability (Fig. 2). Primer set 5 did not result in comparable results possibly due to previously mentioned problems. Although set 6 of the primer was able to generate bands in expected strains, the results obtained were not sufficiently clear for an accurate comparison (Fig. 4). Appropriate bands showed up for JW3914-1 and PN11W-3 from set 6 displaying the retention of the *kanR* gene. However, due to PCR ratio errors, imaging error, or the age of the templates, not all strains showed their expected bands.

In the catalase test, PN11W-3, PN11W-4a, and PN11W-4b strains resulted in expected phenotype of no observable reaction as these strains no longer contained a catalase gene to degrade H_2O_2. Strains PN11W-1 and JW1712 only displayed an observable reaction in the lower 2% concentrated H_2O_2, suggesting that the *KatG* activity may be affected by the concentration of H_2O_2. This observable reaction was delayed was due to lag time required to activate *katG* by the OxyR protein(1,2,3,13). Initially when *E.coli* cells are faced with H_2O_2 challenge and at concentrations lower than 20 μM the *ahpCF* gene products act as a primary scavenger, independent of the OxyR protein, to rapidly break down the toxic chemical. However any higher amounts of H_2O_2 saturates the ahp system and therefore must rely on catalase for clearance (14). When stimulated by a speculated certain threshold of ROS, the OxyR protein induces the activation of the *oxyR* regulon which then allows the production of HPI catalase to protect these strains from ROS. Under the higher concentration of H_2O_2, cells of these strains could not withstand the high amount of ROS and suscumb to death before they can counteract. Another hypothesis is posed that at a high threshold, *katG* cannot be activated (2,10). Since *katE* is permanently induced and does not need the activation of OxyR, strains with *katE* gene, strains JW3914-1 and PN11W-2 immediately and

vigorously react to 30% H_2O_2 to protect the cells from ROS (2).

However, a unique turbidity production reaction has been noted for strain PN11W-4a which has kanamycin resistance gene in place of the *katE* catalase gene. We have put forward the idea that the *kanR* gene is expressed when *katE* locus promoter is expressed even further in the presence of H_2O_2 and that may have caused the "milkiness" as a result of the gene expression. However, in the 2% H_2O_2 test where *katG* locus is supposedly expressed, the same observation was not observed in PN11W-3, possibly due to low promoter expression or to the lower activity of HPI catalase (13). It has been suggested in multiple articles that phosphotransferase systems can be involved in peroxide stress tolerance (15). Kanamycin is an aminoglycoside that inhibit bacterial synthesis; however, the mechanism of aminoglycosides are largely unknown. We do know, however, that the use of antibiotics can sometimes induce increased quantity of reactive oxygen species in the cells exposed to it even though it is not the route that the bacteria is killed (16). The *kanR* gene used in this study encodes an aminoglycoside phosphotransferase that is specialized in kanamycin resistance (17). There may be some undiscovered relationship between antibiotic resistance and hydrogen peroxide synthesis.

In this experiment we sought to confirm the single *katE* and *katG* knockouts and double *katE/katG* knockout generated in a prior work by PCR verification methods, catalase test activity, and survivability at the presence of kanamycin. The results indicated that the single knockouts created were of expected genotypes for *katE* mutations. However, one of the double knockout, PN11W-4a illustrated unexpected kanamycin resistant phenotype which is we confirmed due to a retention of the *kanR* gene on the *katG* locus. Although we confirmed the complete absence of *kanR* gene at the *katE* locus in the double knockout strains, we can only confirm the presence of the *kanR* gene still remaining on the *katG* locus of the PN11W-3 and PN11W-4a strains.

FUTURE DIRECTIONS

There are a number of follow-ups that we can do with our experiment. Although we have confirmed the retention of *kanR* on one of the double knockout strain, future optimized PCR experiments with our designed primers can be done to confirm our results. Validation of the expected gel band pattern of the PN11w-4a strain will fully confirm this strain's genotype and its associated phenotype. If the presumptive double mutant does not exist in our collection of strains, then an attempt to make a new double knockout construct should be made for further experiments to access consequences of altering these genes. Similarly, an electrophoresis gel profile of these 7 strains using our designed primer sets could be generated and analyzed to provide a full comprehensive and concrete evidence of the strains' genotype.

KatE is more protective against sudden exposure to reactive oxygen species. The absolute range of the H_2O_2 concentration where *katG* is expressed is not determined. A range of different H_2O_2 concentrations between 2% to 30% can be exposed to JW-1721 to identify that range. Precise threshold ranges can be identified by the observation of turbidity in H_2O_2-induced reactions. The catalase test could be repeated using cells grown under anaerobic conditions. Genes under the *katE* promoter are constitutively expressed in aerobic conditions; by growing the cells in the absence of oxygen, we can test if there are similar delays in the genes under *katE* gene locus like those observed in the genes under *katG* gene locus. Alternative reporter proteins can be used in these tests.

The effect on the cells' lifespan due to continued genetic engineering on these strains could also be investigated. It seems that from our results, parental strains are more robust in their life span lengths than the strains that were genetically modified.

An investigation in the nature of the reaction between the *kanR* gene product and hydrogen peroxide that was observed in our experiment could be explored. This can be done with a bacteria cell constitutively expressing *kanR* gene encoded aminoglycoside phosphotransferase or the isolated and purified protein product.

ACKNOWLEDGEMENTS

Financial support was provided by the Department of Microbiology and Immunology, University of British Columbia. We would like to thank Dr. William Ramey and Kirstin Brown for their insightful guidance, patient support, and graciously complying with our requests whether it be convenient or not.

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