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Construction of Catalase Double Knockout *Escherichia coli* Strain for Isogenic Strain Comparison Studies of the Role of Catalase

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***Escherichia coli* encodes for two catalases, *katE* and *katG*, that may mediate protection to damage from reactive oxygen species. Prior studies have attempted to assess the contribution of the individual catalases, albeit through comparison of knockout strains on non-isogenic backgrounds. This study aimed to generate a *katE/katG* double knockout strain on the well-characterized BW25113 *E. coli* K-12 strain background through Lambda Red recombination knockout of *katG* on an existing *katE* single knockout strain. Generation of double knockout was successful. The kanamycin resistance cassettes were then removed from the single and double knockouts via FLP-FRT recombination, to allow direct strain comparisons without extraneous phenotypes.**

UV-A mediated killing of pathogens, such as *E. coli*, is believed to arise through the creation of reactive oxygen species (ROS) and their derivatives (4). It is known that catalases convert hydrogen peroxide to water and oxygen, which may confer resistance to the damaging ROS (11). *E. coli* has two catalases, encoded by *katE* and *katG* that are regulated separately (13). Though the catalases are not essential for growth, *katE* product activity does provide protective effects to oxidative stress while *katG* expression is up-regulated by induction with H_2O_2 (13).

Various strains of *E. coli* lacking catalase have been used to analyze the effects of the two catalase genes. However, there has been insufficient direct evaluation between the knockout strains and the starting parental such as in the studies using the B23 strain, which is the parental strain for UM197, but not for UM2 (1, 6). Inconsistencies also exists in the results obtained among the various studies, where there are no consensus on the effect of *katG* single knockout and *katE* single knockout in terms of catalase activity and UV-A survival (1, 5, 6, 10, 12). Also, one prior work that did include a *katE* single knockout of the same parental strain as the *katG* knockout failed to test the strains individually for catalase activity and UV-A survival (13). Another problem is the unnecessary complicating factors added in comparison studies such as using pBAD24-a*katE* for creating double knockout, with the pBAD24 vector complicating analysis (5, 9, 14).

In our experiment, our objective was to develop catalase knockout strains on the well-characterized BW25113 *E. coli* K-12 isogenic strain background. This was proposed as a method to eliminate the extraneous complicating factors that may have caused the discrepancy in prior work. We believe that isogenic comparisons would give more informative data for assessing the protective contributions of the catalase genes.

MATERIALS AND METHODS

Existing *katE* and *katG* single knockout strains were available on the well-characterized isogenic BW25113 *E. coli* K-12 strain background (2). The purpose of this experiment was to create a *katE*/*katG* double catalase knockout strain on the same background. This was done by knocking out *katG* from the existing *katE* knockout strain with kanamycin resistance cassette insertion via the Lambda Red recombination system (mechanism mediated by phage genes on helper plasmid), which allows scarless recombination and generation of knockout (7, 15). The antibiotic cassettes of the *katE*/*katG* double knockout, *katE* single knockout, and *katG* single knockout strains are then removed via the FLP-FRT recombination system (mechanism mediated by the Flippase enzyme on helper plasmid) via FRT regions in the kanamycin resistance cassette to ensure consistency in antibiotic resistance status among the strains (7).

Bacterial strains and plasmid. *E. coli* strains used are listed in Table 1, parenthesis indicate names used in this report. Cultures were grown in 2xYT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl for liquid or with addition of 1.5% agar for plating) at appropriate temperatures with appropriate addition of antibiotics, as indicated below in the growth condition column. Antibiotic concentrations were used as follows: kanamycin at 50 μg/ml and ampicillin at 100 μg/ml. All liquid cultures were grown shaken at 225 rpm. Overnight cultures were grown from glycerol stock inoculated to 5 ml liquid medium.

DNA extraction of pKD13, pKD46, and BT340. Plasmid pKD13 was isolated using Fermentas GeneJet™ Plasmid Miniprep kit as described (8). Plasmids pKD46 and BT340 were isolated using QIAGEN QIAquick™ Miniprep kit as described (16).

PCR amplification of *katG* kanamycin resistance targeting cassettes. Table 2 lists the kanamycin resistance cassette primers as described (2) (ordered from Integrated DNA Technologies). Primers contain complementary sequences to the kanamycin resistance gene in the pKD13 template and to the targeted gene (*katG*). PCR was done in 50 ul reaction (2.5 U Taq polymerase, 1.0 mM each of

TABLE 1: *E. coli* strains and plasmids used in this study.

Strain	Genotype	Growth Condition	Source
BW25113 (parental)	F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, rph-1, Δ(rhaD-rhaB)568, hsdR514	37°C	University of British Columbia, Department of Microbiology & Immunology, MICB 421 laboratory stock
BW25141/pKD13 (kanamycin resistance cassette template)	F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), Δ(phoB-phoR)580, λ-, galU95, ΔuidA3::pir+, recA1, endA9(del-ins)::FRT, rph-1, Δ(rhaD-rhaB)568, hsdR514, pKD13	37°C , kanamycin	University of British Columbia, Department of Microbiology & Immunology, MICB 421 laboratory stock
BW25113/pKD46 (Lambda Red helper plasmid)	F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, rph-1, Δ(rhaD-rhaB)568, hsdR514, pKD46	30°C, ampicillin	University of British Columbia, Department of Microbiology & Immunology, MICB 421 laboratory stock
JW1721-1 (*katE* single knockout strain)	F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, ΔkatE731::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514	37°C , kanamycin	University of British Columbia, Department of Microbiology & Immunology, MICB 421 laboratory stock
JW3914-1 (*katG* single knockout strain)	F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, rph-1, Δ(rhaD-rhaB)568, ΔkatG729::kan, hsdR514	37°C , kanamycin	University of British Columbia, Department of Microbiology & Immunology, MICB 421 laboratory stock
BT340 (FLP recombinase helper plasmid)	F-, Δ(argF-lac)169, φ80dlacZ58(M15), glnV44(AS), λ-, rfbC1, gyrA96(NalR), recA1, endA1, spoT1, thi-1, hsdR17, pCP20	30°C, ampicillin	University of British Columbia, Department of Microbiology & Immunology, MICB 421 laboratory stock
PN11W-1 (*katE* single knockout strain with kanamycin resistance cassette removed)	F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, ΔkatE731, rph-1, Δ(rhaD-rhaB)568, hsdR514	37°C	Created in this study
PN11W-2 (*katG* single knockout strain with kanamycin resistance cassette removed)	F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, rph-1, Δ(rhaD-rhaB)568, ΔkatG729, hsdR514	37°C	Created in this study
PN11W-3 (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	37°C, kanamycin	Created in this study
PN11W-4a/b (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	37°C	Created in this study

forward and reverse primer, 200 mM dNTPs, 1 pg pKD13 template DNA) with 30 cycles (94°C for 30 s, 59°C for 30 s, 72°C for 2 min). Product was verified to be of 1403 bp via standard 1% gel electrophoresis with ethidium bromide post-stain, and used as-is for electroporation.

Preparation of competent cells. Competent cells for electroporation were generated following a published protocol adapted as follows (3). Liquid culture of 30 ml were inoculated with 1 ml overnight culture and allowed to grow to an OD_{600} of 0.6. Twenty milliliters of culture were pelleted at 1000 x g for 15 min, washed twice with 20 ml 10% ice cold glycerol, once with 1 ml 10% glycerol and re-suspended in 200 µl 10% glycerol.

Electroporation of plasmid or *katG* targeting DNA. Electroporations were carried out following the manufacturer protocol adapted as follows (3). Competent cells were made and 60 µl of the re-suspension were electroporated on BioRad Micropulser™ on the default *E. coli* setting with 400 ng of either isolated plasmid or targeting cassette and outgrown for 1 hr with SOC media (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose). Selections were done by plating up to 100 µl of the outgrown culture on the appropriate solid medium.

FLP recombinase removal of kanamycin resistance cassettes. Kanamycin resistance cassettes in the JW1721-1 and JW3914-1 strains were removed following a published protocol adapted as follows (7, 15). BT340 plasmid was electroporated into competent cells and selected for on ampicillin plates at 30°C to allow recombination. Successful recombinants were screened in triplicate by colony grid streaking on three plates containing either ampicillin, kanamycin, or no antibiotics that were grown at 42°C overnight to remove the cassette and inhibit the temperature-sensitive plasmid to select for clones with no antibiotic resistance.

Lambda Red recombination for generation of *katE*/*katG* catalase double knockout. Lambda Red recombination was carried out following protocols adapted as follows (2). The JW1721-1 strain with existing kanamycin resistance cassette removed was made

TABLE 2: PCR primers to generate the kanamycin resistance cassette targeting *katG*.

Forward Primer	5′-CTAACGCTGTGTATCGTAACGGTAACACT GTAGAGGGGAGCACATTGATGATTCCGGGG ATCCGTCGACC-3’
Reverse Primer	5′-CCAGCAAGCAGCCGCTGAACGGGGTCAG ATTACAGCAGGTCGAAACGGTCTGTAGGCT GGAGCTGCTTCG-3’

competent and electroporated with pKD46. Transformants were then selected for on ampicillin plates incubated at 30°C. Overnight culture of the resulting JW1721-1/pKD46 strain were supplemented with 1 mM L-arabinose to induce the Lambda Red system gene expression, made competent, electroporated with the *katG* kanamycin resistance cassette, outgrown for 2 hrs at 37°C and selected by growth on kanamycin plates at 37°C to remove the temperature-sensitive pKD46 plasmid. The kanamycin resistance cassette was subsequently removed after PCR verification.

PCR verification of the recombination of *katE*/*katG* catalase to form a double knockout. Successful recombination was assessed via colony PCR with primers as listed in Table 3 (ordered from Integrated DNA Technologies). Primers k1and k2, which lies within the kanamycin resistance cassette, were used for the same verification procedure in previous studies (2). Primers U and D flanks the inserted kanamycin resistance cassette and were planned by using the Primer3Plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) as previously described (2). PCR was done in 20 ul reaction (2.5 U Taq polymerase, 1.0 µM each of forward and reverse primer, 200 µM dNTPs, 1 pg pKD13 template DNA) with 35 cycles. The conditions for the k1/U primer set was 94°C for 30 s, 51°C for 30 s, 72°C for 2 min, and for k2/D primer set was 94°C for 30 s, 48°C for 30 s, 72°C for 2 min. Products were verified to be of expected sizes: (set 1) 930 bp, (set 2) 1315 bp, via standard 1% gel electrophoresis with ethidium bromide post-staining.

RESULTS

FLP recombination of *katG* and *katE* single knockouts. FLP recombination was carried out with the helper plasmid BT340. Recombination was successful in removing the kanamycin resistance cassette on first incubation at 42°C, as evidenced by the absence of growth on kanamycin plates (data not shown). However, only after second streaking and incubation at 42°C was the helper plasmid removed, as evidenced with the absence of growth on ampicillin plates (data not shown). Thus, *katG* and *katE* single knockouts with no antibiotic resistance were obtained.

TABLE 3: Test primers for PCR verification of Lambda Red recombination.

Primer Label	Primer Sequence	Annealing Temperature
k1 (set 1)	5’-CAGTCATAGCCGAATA GCCT-3’	Tm= 51.10°C
k2 (set 2)	5’-CGGTGCCCTGAATGAAC TGC-3’	Tm= 56.20°C
U (set 1)	5’-AGCCGTGAAGGAGTGAAA GA-3’	Tm=53.09°C
D (set 2)	5’-ACTGACGCCGGAGAGGAT TG-3’	Tm= 56.14°C

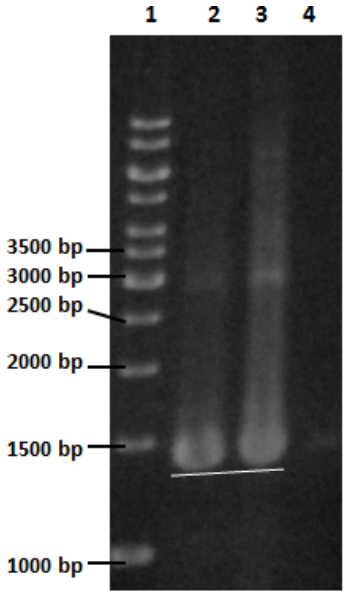

FIG. 1. Identification of the kamamycin resistance targeting cassette by 1% agarose electrophoresis. Lane 1: GeneRuler™ 1 kb DNA ladder; Lane 2: PCR product of kanamycin resistance cassette with targeting arms (2 ul DNA); Lane 3: PCR product of kanamycin resistance cassette with targeting arms (20 ul DNA); Lane 4: Negative control. The band above white line indicates expected product of 1403 bp.

PCR generation of kanamycin resistance cassette with targeting arms. The kanamycin resistance cassette in pKD13 plasmid was amplified with the addition of the targeting arms for homologous recombination. The product amplification was successful showing the expected band size of 1403 bp product (Fig. 1). Approximation of the DNA quantity in the gel correlated with spectrophotometer readings, as expected. Banding pattern does show extraneous PCR by-products.

Lambda Red recombination of *katE* knockout for creation of double knockout. To remove the *katG* gene from the *katE* knockout strain, the Lambda Red recombinase helper plasmid, pKD46, was first transformed into the strain. The transformation of pKD46 was successful as evidenced by the presence of

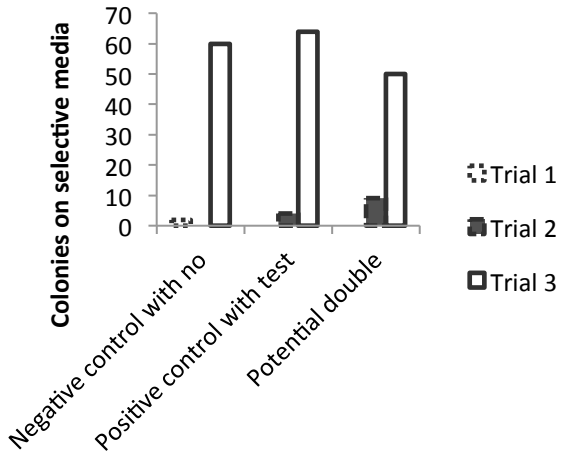

FIG 2. Number of colonies observed in selective plates following each attempt to select a double knockout. The positive control was the empty vector. Numbers obtained were by manual colony counts.

growth on ampicillin plates (data not shown). In order to remove the *katG* gene, Lambda Red recombination of the *katE* knockout strain with *katG* targeting kanamycin resistance cassette was performed. Three trials were attempted. The recombination was not successful for trial one because of the lack colony growth. Trial three was also unsuccessful because of an excessive number of colonies in the negative control with no kanamycin resistance cassette. Trial two showed a number of significant colonies on selective media and no growth in the negative control even though positive control is lacking in growth (Fig. 2). Surprisingly, colony size in all trials was varied (data not shown). Due to the above inconsistencies, the quality of Lambda Red recombination data was not ideal.

PCR verification of catalase double knockout. To verify the removal of the *katG* gene from the *katE* single knockout, PCR was performed on the potential Lambda Red recombination double knockout positives with two primer sets: k1/U, and k2/D. The amplification was successful for all six potential positives tested, as shown by the presence of the expected band size of 930 bp for k1/U primer set (Fig. 3), and 1315 bp for k2/D primer set (Fig. 4). The negative control of the parental BW25113 strain showed an absence of the product bands, as expected. However, there were extra band sizes of 750 bp, 800 bp and 1500 bp present in all the samples with primer set k2/D (Fig. 4), and an extra band size of 1300 bp with k1/U primer set (Fig. 3). The extra banding pattern was not expected.

FLP recombination of the *katG/katE* double knockout. Similarly to the single catalase knockout strains, the kanamycin resistance cassette was successfully removed from the catalase double knockout after two 42°C incubations (data not shown).

DISCUSSION

The aim of this study was to create a *katG/katE* catalase double knockout strain of *E. coli* on the same BW25113 strain background as the available *katG* and *katE* catalase single knockouts whilst removing antibiotic resistance.

In the Lambda Red recombination procedure, sufficient numbers of growing colonies were only obtained when the outgrowth time and temperature after electroporation was increased (from 30°C for 1 hr to 37°C for 2 hrs). This may be explained by the rarity of such recombination events resulting in need for extended growth. However, recombination rates in this experiment were much lower than indicated by previous studies (2). The targeting DNA quantity was confirmed to be sufficient (Fig. 1) and no attempt was made to purify the PCR product as neither plasmid template nor by-products could generate a viable recombination. It may be that the competency of the cells were not optimal, which may explain the lowered growth rate in the positive controls (Fig. 2). As mentioned in the previous section, there were different sizes of colonies present following selective plating of the electroporated cells. Interestingly, PCR verification showed only the larger-sized colonies were successful recombinants (data not shown). Though no prior studies have indicated similar results from Lambda Red recombination, it is suspected those off-target

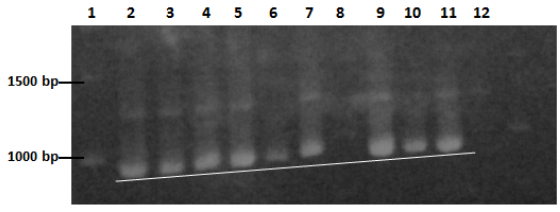

FIG. 3. PCR verification of red recombination with k1/U primer. Lanes 1 and 8: GeneRuler™ 1 kb DNA ladder; Lanes 2-7: Potential double knockouts; Lanes 9-11: Positive control (JW3914-1); Lane 12: Negative control (Parental BW25113). The band above white line indicates expected product of 930 bp.

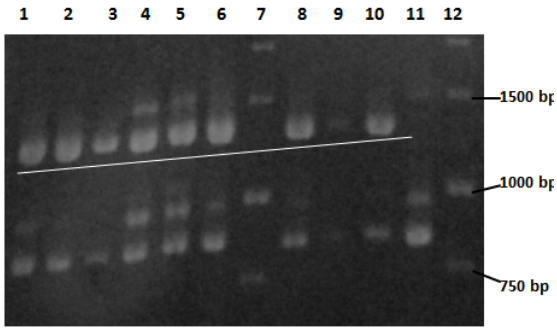

FIG. 4. PCR verification of red recombination with k2/D primer. Lanes 1-6: Potential double knockouts; Lanes 7 and 12: GeneRuler™ 1 kb DNA ladder; Lanes 8-10: Positive control (JW3914-1); Lane 11: Negative control (Parental BW25113). The band above white line indicates expected product of 1315 bp.

recombinations mediated by the *E. coli* existing recA system may have caused decreased viability and thus, decreased the colony size for those colonies.

PCR verification of the *katG* red recombination showed extra banding patterns apart from the expected products (Fig. 3). Likely there were mis-priming involved due to priming during set-up of the tests or priming at a permissive temperature. But due to time constraints, a temperature gradient PCR was not performed. Nevertheless, verification was still successful since these extra bands were present in both the positive and the negative PCR controls, indicating background-specific amplification, while product bands were not present in the negative control. Unfortunately, no PCR verification was done to confirm the continued existence of the *katE* knockout.

FLP recombination removal of the kanamycin resistance cassette worked with high efficiency, as expected from previous studies (7). Two overnight incubations, instead of one, however, were required to remove the ampicillin resistance, perhaps indicating the presence of high concentrations of the helper BT340 plasmid. Again, no PCR verification was done to confirm the continued existence of the knockouts. This missing verification is an issue because the original selective factor, the kanamycin resistance cassette, was firstly removed prior to Lambda Red recombination to allow selection, and secondly after recombination, for antibiotic resistance consistency among the strains. It is assumed that proper strain tracking, proper aseptic techniques, the low likelihood of spontaneous mutations, and the low rate of erroneous clone selection would eliminate inappropriate constructs and contaminations.

Taking into account the positive results of the PCR verification, this study has successfully generated a *katE/katG* catalase double knockout strain. This study has also successfully removed the kanamycin resistance cassette from the *katE* and *katG* single knockout strains in addition to the double knockout strain.

FUTURE EXPERIMENTS

The strains created in this study (*katE/katG* catalase double knockout strain, *katE* single knockout strain, *katG* single knockout strain, all without kanamycin resistance), after further verification of the absence of *katE* and catalase activity, can be used for direct comparison with the wildtype BW25113 parental strain. Assessments may include catalase activity tests and UV-A survival tests to observe the individual and combined contributions of *katE* and *katG* to ROS resistance.

Future investigations looking into other genes involved in ROS protection would benefit from detailed trouble-shooting for optimization of the Lambda Red recombination knockout method. PCR verification should be supplemented or substituted with sequencing verification for accurate assessment of recombination.

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