Adaptive Kanamycin Resistance Induced by the Stringent Response in *Escherichia coli* is Not Dependent on *katE* and *katG*

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Treatment of *Escherichia coli* with sub-inhibitory levels of valine has been observed to confer kanamycin resistance; however, the role of catalase in this resistance is unknown. In this study, the role of catalase activity in increased resistance to kanamycin due to valine-induced amino acid starvation was investigated. *E. coli* K-12 wild-type strain and mutants Δ*katE*, Δ*katG*, and Δ*katE*/Δ*katG* were used to assess kanamycin resistance after their corresponding deletions were validated by PCR. Once starved using an excess of valine, growth was monitored by turbidity and catalase activity was measured by a floating disc assay. Antibiotic resistance was assessed using transient antibiotic resistance assays and minimum inhibitory concentration tests. Valine-induced amino acid starvation resulted in short-term antibiotic resistance, but did not confer long-term resistance. Comparing the adaptive resistance between the wild-type, Δ*katE*, Δ*katG*, and Δ*katE*/Δ*katG* strains as assessed by transient resistance assay and minimum inhibitory concentration, no loss in adaptive resistance was observed in any deletion mutant. The catalase activity measured by floating disc assay showed no difference between the control and starved cultures; however, the WT and Δ*katG* had significantly higher catalase activity compared to Δ*katE*, while Δ*katE*/Δ*katG* had no activity. Taken together, these findings suggest the catalases encoded by *katE* and *katG* were not responsible for conferring the short-term adaptive kanamycin resistance.

Stringent response is required for bacterial survival under environmental pressures such as nutrient limitation and oxidative stress. Valine-induced amino acid starvation activates the stringent response in *Escherichia coli*, and has been observed to increase adaptive resistance to antibiotics (1). Adaptive resistance is characterized as the ability of bacteria to survive antibiotic challenges without mutation, and is viewed as the consequence of bacterial responses to an environmental signal (2). The involvement of capsule thickening (3), efflux pump regulation (4), and porin content (5) have all been previously studied in relation to adaptive kanamycin immunity. However, the role of the catalase genes *katG* and *katE*, encoding hydroperoxidase I (HPI) and hydroperoxidase II (HPII) respectively, in adaptive kanamycin resistance is poorly characterized. The catalases, hydroperoxidase I and II, along with superoxide dismutases (SOD) are enzymes known to counteract oxidative stress. Catalase activity has been shown to increase in *E. coli* following valine-induced amino acid starvation (6).

Activation of stringency in response to amino acid starvation in Gram-negative bacteria involves a change from transcription of genes involved in cell growth to genes involved in survival (1). Transcriptional switching is mediated by the alarmone guanosine 5′-(tri)diphosphate ((p)ppGpp). (p)ppGpp levels are regulated by RelA and SpoT. When reduced amino acid levels are sensed during translation, (p)ppGpp is synthesized by ribosome-associated RelA while the (p)ppGpp hydrolyase activity of SpoT decreases. (p)ppGpp inhibits the synthesis of tRNA and rRNA and in turn prevents translation of new peptides (7). Additionally, the shift of transcription away from stable RNA allows for the cell’s transcriptional machinery to focus on a wider array of genes, including those associated with the onset of the stringent response or those required for entry into stationary phase (8). Antibiotic induced stress has been shown to induce oxidative killing by increasing the amount of reactive oxygen species (ROS) such as hydrogen peroxide, superoxide radicals, and hydroxyl radicals (9). ROS can damage methionine and cysteine residues, iron-sulfur clusters, and DNA directly through the Fenton reaction. Some bacteria can combat oxidative stress by hydrolyzing hydrogen peroxide in the reaction $H_2O_2 \rightarrow H_2O + \frac{1}{2}O_2$.

In this experiment, we examined the role of the catalase genes *katE* and *katG* in adaptive kanamycin resistance during the stringent response. The adaptive transient resistance and minimum inhibitory concentration (MIC) of kanamycin of the deletion mutants Δ*katE*, Δ*katG* and Δ*katE*/Δ*katG* following valine-induced amino acid starvation were compared to WT *E. coli*. Differences in kanamycin resistance between the Δ*katE*, Δ*katG* and Δ*katE*/Δ*katG* observed during amino acid starvation may indicate a role for the catalase genes in adaptive antibiotic resistance. To further link catalase to kanamycin resistance, a catalase assay was performed comparing the catalase activity of the amino acid starved and replete *E. coli* cultures. Differences in catalase activity observed during replete and amino acid starvation may further confirm the role for the specific catalase genes in adaptive antibiotic resistance.
MATERIALS AND METHODS

Bacterial strains and growth conditions. E. coli K-12 parental strain BW25113 (WT), P1N1W-1 (ΔkatE), P1N1W-2 (ΔkatG), and P1N1W-4b (ΔkatE/ΔkatG) were obtained from the MICB 421 culture collection in the Microbiology and Immunology Department of the University of British Columbia. All mutant strains were deletion mutants without a kanamycin resistance gene. Stock cultures were streaked on tryptic soy agar plates (1.5% w/v casein peptone, 0.5% w/v soya peptone, 0.5% w/v NaCl, 1.5% w/v agar in distilled water). One colony from each strain was inoculated overnight at 200 rpm and 30°C Hui et al (11) in 25 ml of M9 minimal salt media (0.005% w/v NaCl, 0.7% w/v NaHPO4, 0.3% w/v KH2PO4, 0.1% w/v NH4Cl, 0.02% w/v MgSO4·7H2O, 0.2% w/v glycerol in distilled water).

Valine-induced amino acid starvation and sampling. Overnight cultures were used to inoculate cultures of 120 ml M9 media to a final OD600 of 0.15. Start cultures were incubated at 37°C for 1 h in shaking water baths at 200 rpm. Cultures were then split into two flasks each with a total volume of 50 ml, in which one contained 0.1 mg/ml of valine. All cultures were incubated for 1.5 h at 37°C and 200 rpm in shaking water baths (6). During amino acid starvation, 4 ml samples were taken every 0.5 h and measured at OD600 using Spectronic 20 spectrophotometer to monitor cell growth. 10 ml samples were taken from each flask at the beginning and end of valine treatment for follow-up catalase activity, minimum inhibitory concentration, and transient antibiotic assays.

Confirmation of KO strains. PCR verification of katE and katG in WT, ΔkatE, ΔkatG, and ΔkatE/ΔkatG was performed via colony PCR. Each colony was resuspended in 10 µl of DEPC water of which 1 µl was used for the subsequent PCR reaction. The primers (KatE-F-UHHK-12W, KatE-r-UHHK-13W, KatG-F-UHHK-13W and KatG-r-UHHK-13W) designed by Hui et al (11) were used to verify the deletion mutants. PCR was performed with a final volume of 25µl with each reaction containing: 1x of 10x Hotstart Buffer (Invitrogen, Cat. No. 18067-013), 0.4 µM of each of the forward and reverse primers, 0.4 mM dNTP, 1 mM MgCl2, and filled to a final volume of 25 µl with DEPC-H2O. The PCR cycle consisted of an initial 95°C denaturation for 2 min followed by 35 cycles of 45 sec denaturation at 95°C, 45 sec annealing at 58°C, and 2.5 min extension at 72°C. A final extension step at 72°C for 8 min was performed before storage at 4°C. The PCR products and an E-gel High Range DNA Marker (Invitrogen, Cat. No. 12352-019) were run on a 1% (w/v) agarose gel in 1X TBE buffer (0.1 M Tris, 0.1 M boric acid and 2mM EDTA). The agarose gel was stained for 1 h in 0.5 µg/ml ethidium bromide and visualized on a Multimage light cabinet.

Relative catalase assay. The floating disc assay was adapted from Hsieh et al (12). Each of the four strains, with and without valine-induced amino acid starvation, were tested for catalase activity. 10 µl of standardized 0.450 OD600 cell culture was loaded onto 5 mm filter discs (Millipore, Cat. No. 01730). After 10 sec, the soaked filters were placed in a 100 ml beaker with 50 ml of 1% (v/v) hydrogen peroxide (H2O2) solution, made from 35% (v/v) stock (Alfa Aesar, Cat. No. L14000). Catalase activity was determined as the inverse of the time needed for the filters to float to the top. Discs which clung to the side of the beaker or sank back to the bottom were discarded. The experiment was repeated in triplicate.

Kanamycin minimum inhibitory concentration (MIC) assay. A 20 mg/ml kanamycin stock solution in distilled water was prepared and sterilized using a 0.22 µm filter. Sterile 96-well polyethylene microtitre round bottom plates were pre-plated with kanamycin in M9 media in serial dilution from 16 to 0.25 µg/ml (6). Cell samples were added to the plates 1.5 h post starvation treatment, and triplicates were performed for each cell strain. 50 µl of standardized culture was added to 150 µl of diluted antibiotics to obtain a final cell concentration of 0.025 OD600 and an appropriate kanamycin concentration in 200 µl total volume. Three controls were prepared for each condition: media control which contained only media; negative control, which contained culture and 1 mg/ml of kanamycin; and positive control, which contained culture but no antibiotics. Four plates were set up in total for each experiment to accommodate all conditions. The plates were incubated at 37°C for 24 and 96 h and the MIC was determined by observing the well with the lowest antibiotic concentration that inhibited bacterial growth.

Transient antibiotic resistance assay. Sterile 96-well clear flat bottom tissue-culture treated microplates with identical layout as the MIC plates were inoculated with 125 µl of standardized 0.015 OD600. The plates were read by Bio-Rad Microplate Reader model 3550 prior to incubation to obtain initial turbidity in each well. Plates were incubated for 3 h at 37°C on a 200 rpm shaker. During this time, turbidity readings via the plate reader were taken at 30 min intervals. These OD600 measurements were used to calculate the change in turbidity over time.

Statistical analysis. 1-way ANOVA was performed to determine if the difference in catalase production under control and starved conditions was significant. Microsoft Excel 2007 was used in data analysis.

RESULTS

PCR verification of deletion mutants. Primers designed for the PCR amplification of either katE or katG were used to confirm the deletion of the catalase genes in the mutant strains. Absence of bands upon electrophoresis of the PCR product at ~2.3 kb and ~2.2 kb corresponding to katE and katG, respectively, were used to confirm each deletion (Fig. 1). The WT strain displayed bands corresponding to katE at 2.3 kb and katG at 2.2 kb. Fainter bands observed in lanes 2, 3, and 6 are likely due to non-specific binding of the primers. Absence of a band at 2.3 kb was used to confirm the deletion of katE from the ΔkatE strain while absence of a band at 2.2 kb was used to confirm the deletion of katG from the ΔkatG strain. The ΔkatE/ΔkatG double deletion mutant was confirmed by the absence of both 2.3 kb and 2.2 kb bands. These results are consistent with previous reports (11).

Catalase activity was not affected by valine-induced starvation in mutant strains. Catalase activity of bacterial cells was assessed using a floating disc assay (Fig. 2). Prior to performing the assay, starvation was confirmed by a reduced growth rate in the valine-treated culture. The WT strain was expected to have a combination of catalase activity from katE and katG, since it contains both of these genes. However, the WT intrinsic catalase activity was comparable to the ΔkatG strain (Fig. 2). Therefore, we reasoned that katE was the dominant gene attributing to intrinsic catalase activity in the WT strain. The intrinsic catalase activity of the ΔkatE strain was 3.9-fold lower than the WT and 3.8-fold lower than the ΔkatG strain (Fig. 2). As katE is reported to be induced by RpoS (9) in response to amino acid starvation, it was expected that the WT and ΔkatG strains would display an increase in catalase activity under starvation. However,
Starvation did not induce kanamycin resistance after long-term incubation. The MIC assay was used to evaluate the development of long-term resistance to kanamycin for WT, ΔkatE, ΔkatG and ΔkatE/ΔkatG of E. coli was evaluated (Table 2). Under control conditions, growth inhibition of WT and ΔkatE/ΔkatG strains required a 2-fold increase in kanamycin concentration than of ΔkatE and ΔkatG strains. The MIC was reduced for all strains under starvation conditions. Starvation of the WT strain resulted in an 8-fold decrease in the inhibitory concentration of kanamycin compared to the control. In the ΔkatE and ΔkatG strains, starvation reduced the inhibitory
concentration by 16-fold, while in ΔkatE/ΔkatG the inhibitory concentration was reduced by 32-fold.

**TABLE 1.** Effect of starvation pre-treatment on short-term kanamycin resistance in *E. coli* K-12 ΔkatE, ΔkatG and ΔkatE/ΔkatG.

<table>
<thead>
<tr>
<th>[Kanamycin (μg/mL)]</th>
<th>Time of growth inhibition (h)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>ΔkatE</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
</tr>
<tr>
<td>8</td>
<td>1.0</td>
</tr>
<tr>
<td>16</td>
<td>0.5</td>
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**DISCUSSION**

Previous studies have shown that amino acid starvation confers kanamycin resistance in *E. coli* (6). This study attempted to test whether short-term resistance is due, in part, to the expression of the catalase gene katE, which is induced by the onset of stringent response (10). Mutant strains containing deletions in katE, KatG, or katE/katG were compared to WT with respect to catalase activity and transient resistance to kanamycin in response to valine-induced amino acid starvation. The results of this study suggest no correlation between the expression of katE or katG and the adaptive resistance conferred by amino acid starvation.

The WT strain displayed a 1.2-fold increase in catalase activity under starvation compared to the control; however, this result is not consistent with the substantial increase in catalase activity under starvation seen previously (6). The increase in catalase activity may be attributed to katE induction by sigma factor RpoS (9), since high catalase activity was observed in the ΔkatG strain compared to ΔkatE. Therefore, KatE is likely responsible for majority of catalase activity in the WT. Conversely, starvation led to a 1.9-fold decrease in catalase activity in the ΔkatE strain. KatG is expressed in response to low concentrations of H₂O₂ generated during exponential phase (13); hence, the observed decrease in catalase activity in the starved ΔkatE culture may be due to a slower growth rate compared to the control. If the former mechanism is true, then the substantial increase in KatE activity induced by stringent response may be masked by a decrease in KatG due to attenuated growth in the starved WT culture. The result would be the small observed difference of catalase activity between the control and starved WT. However, the ΔkatG strain, in which the activity is attributed solely to katE, did not undergo an increase in catalase activity akin to or greater than the WT. This questions the validity of the proposed mechanism and highlights that compensation for katG deletion in *E. coli* is not fully understood. Thus, the catalase assay demonstrated no correlation between starvation and increased catalase activity across the strains tested.

Since RpoS is known to induce katE expression (9), strains with an intact katE (WT and ΔkatG strains) were expected to have greater short-term adaptive kanamycin resistance conferred by starvation compared to strains lacking katE (ΔkatE and ΔkatE/ΔkatG strains). Interestingly, all four strains demonstrated a similar level of kanamycin resistance under starvation as determined by time of deviation from the normal growth rate. Although stringent response to amino acid starvation confers short-term adaptive kanamycin resistance, the observed kanamycin resistance is independent of the presence or absence of either katE or katG. The results suggest that stringent response-dependent kanamycin resistance may not rely on oxidative stress reduction mechanisms. Catalase may supplement the removal of H₂O₂ produced during kanamycin-induced killing but is not the dominant mechanism for survival. Additional enzymes such as SOD may be more prominent in the breakdown of ROS. It is likely that other resistance mechanisms such as capsule thickening (3), efflux pump up-regulation (4), and membrane porin content (5) play a larger role in adaptive kanamycin response.

The transient resistance assay used in this study had a range of 3 hours; therefore, potential growth inhibition due to effects of kanamycin occurring after 3 hours in the starved cultures is not known. As a result, there was no comparison of time required for growth deviation between the control and starved cultures. While the data (Fig. 3) does not suggest short-term kanamycin induced inhibition of the starved cultures, there is a possibility that the growth will deviate from the normal growth rate after 3 hours for tested concentrations of kanamycin. Using kanamycin concentrations above 16 μg/ml is an alternative to observe the adaptive resistance within the 3-hour timeframe.

Although long-term resistance evaluated using the MIC assay were shown to be different between strains, a decrease of 8 to 36-fold in kanamycin resistance was observed in all starved cultures compared to control. Contrary to the short-term transient assay results, lower concentrations of kanamycin were required for long-term growth inhibition of *E. coli* after amino acid starvation. The results indicate that cells under the starvation condition may be more prone to cell lysis, resulting in fewer cells being visible in the MIC plate after a long period of time. A long-term antibiotic resistance assay could test for a decrease in turbidity over a large time interval to pinpoint the loss of adaptive resistance conferred by stringent response (14).

In addition, oxidative stress was not directly measured in our study, nor was the role of SOD in reducing oxidative stress in the *E. coli* strains examined. Despite catalase activity being demonstrated to be significantly different between strains (Table 2), there is a possibility that oxidative stress caused by kanamycin was scavenged by SOD, thereby compensating for the difference in catalase activity. Previous studies found that KatG was not necessary for defence against antibiotics (15). An assay
assessing the degree of oxidative stress exhibited by each strain should be performed. Although catalase is not responsible for transient kanamycin resistance, it does not exclude the role of ROS in the bactericidal effects of kanamycin.

In conclusion, the results of this study indicate no difference in short-term and long-term adaptive kanamycin resistance between WT strain and mutants strains lacking the catalase genes katE and/or katG. Additionally, the conferred kanamycin resistance was not caused by catalase, as the catalase activity remained consistent in control and starved cells. As such, the analysis of amino acid starvation in katE and KatG mutants in E. coli strains indicated no relationship between catalase gene deletion and loss of adaptive kanamycin resistance. The study concludes that the breakdown of hydrogen peroxide by catalase is not the mechanism responsible for adaptive kanamycin resistance conferred by stringent response.

FUTURE DIRECTIONS
The results indicate that there is no correlation between short-term adaptive kanamycin resistance conferred by stringent response and the presence of katE and katG. However, the 3-hour duration of the transient assay is insufficient in comparing kanamycin resistance between the starved and control cultures. Future experiments could include a long-term resistance assay performed in previous publications, where turbidity readings are taken after 24 hours (14). With a longer incubation time, growth inhibition can be determined in both control and starved cultures, allowing a better comparison of starvation-induced kanamycin resistance. Furthermore, a difference in growth inhibition time may be observed between starved mutant and WT strains.

Due to the low sensitivity of the floating disc catalase assay, small fluctuations in catalase activity between cultures under control and starved conditions were difficult to differentiate. Trials are performed one at a time, adding to the high variability of each replicate. Use of a colorimetric assay, such as the amplex red stain assay (16, 17), would allow for more precise evaluation catalase activity and its involvement in stringent response. In addition to limiting the external variables, a colorimetric assay would allow for greater throughput and more replicates.

The role of SOD in adaptive kanamycin resistance conferred by stringent response needs to be elucidated. An experiment consisting of valine-induction and transient resistance assays, could be used to evaluate adaptive resistance due to stringent response in SOD deletion mutants. Through such methods, the importance of overcoming kanamycin-induced oxidative killing to adaptive resistance could be assessed. This is necessary to fully investigate the relationship between oxidative stress and adaptive antibiotic resistance.

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