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# Antibiotic Tolerance in *Escherichia coli* Under Stringent Response Correlates to Increased Catalase Activity

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**Bactericidal antibiotics are known to induce bacterial cell death through the production of reactive oxygen species, which can be neutralized by bacterial oxidative stress control proteins such as catalases. In *Escherichia coli*, the *katE* gene encoding catalase is induced by the stringent response pathway. Stringent response is often activated under nutrient and amino acid starvation such as isoleucine deficiency. This study examines stringent response mediated catalase production and its role in antibiotic tolerance. In *E. coli* K-12, and the K-12 deletion mutant *rel*A and double deletion mutant *rel*A and *spo*T, isoleucine starvation was achieved by incubation with excess valine. Catalase production was examined through a floating disk assay, while susceptibility to kanamycin was determined through a bacterial killing assay as well as a XTT viability assay. Results showed that under starved conditions, wild-type *E. coli* had higher catalase production as well as kanamycin tolerance compared to the mutant strains.**

In response to environmental stress such as nutrient-limited conditions, bacteria downregulate transcription of genes for cell growth and upregulate those required for survival. This physiological reprogramming is known as stringent response (1–3). In *Escherichia coli*, stringent response is activated due to production of high levels of guanosine-3',5'-tetraphosphate (ppGpp) and guanosine-3',5'-pentaphosphate (pppGpp) mediated by RelA and SpoT (3). In *E. coli*, RelA synthesizes (p)ppGpp, while the role of SpoT is less clear. SpoT may hydrolyze (p)ppGpp, but in Δ*rel*A mutants, (p)ppGpp is still synthesized through SpoT activity (4). (p)ppGpp directly interacts with RNA polymerase, repressing rRNA synthesis and affecting gene transcription (4).

In a stringent state, *E. coli* displays higher tolerance to many classes of antibiotics. One possible explanation is that many antibiotics only target proliferating cells. However, recent studies indicated drug-induced killing is related to generation of reactive oxygen species, including superoxide ($O_2^{\bullet -}$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals (OH•), suggesting oxidative stress control may play a role in antibiotic tolerance(5, 6). Under normal growth conditions, superoxide dismutases and catalases neutralize superoxide and hydrogen peroxide respectively; no known enzymatic reaction neutralizes hydroxyl radicals. Dysregulation of oxidative stress control under drug-induced stress results from increased aerobic respiration and destabilization of iron-sulfur clusters in dehydratase enzymes. Increased aerobic respiration generates excess superoxides that cannot be controlled by homeostatic levels of superoxide dismutase. These superoxides reduce the iron in iron-sulfur clusters to release ferrous iron, generating hydroxyl radicals through the Fenton reaction (5).

Resistance studies in *Pseudomonas aeruginosa* suggested antibiotic tolerance during stringent response was due to upregulation of oxidative stress control proteins (7). Though wild-type *P. aeruginosa* was antibiotic resistant during stringent response, resistance in a Δ*rel*A/Δ*spo*T *P. aeruginosa* mutant was considerably reduced. This was subsequently linked to decreased levels of antioxidant proteins, such as catalase, coupled with increased production of 4-hydroxy-2-alkylquinolones (HAQs), a group of pro-oxidant proteins. Lower levels of catalase and superoxide dismutase were observed in a mutant lacking stringent response. Furthermore, a triple knockout mutant lacking *rel*A, *spo*T and *pqs*A - a gene for HAQ biosynthesis - showed wild type levels of antibiotic tolerance and wild type levels of hydroxyl radicals. Conversely, overexpression of HAQs in wild-type *P. aeruginosa* led to an increase in antibiotic killing (7).

It is unclear if similar mechanisms of oxidative stress control underlie the antibiotic tolerance observed during stringent response in other species such as *E. coli.* Although *E. coli* lacks HAQs, oxidative stress control systems are similar to those found in *P. aeruginosa*. *E. coli* expresses several antioxidant proteins, including a constitutively expressed *kat*G and a stringent response-induced *kat*E, both encoding for catalase (8). However, stringent response studies were difficult to carry out previously, as Δ*rel*A/Δ*spoT* mutants contained additional metabolic deficiencies, making it difficult to compare to wild-type strains. Recently, a double deletion mutant generated via the λred recombination system (1) was isolated, producing a strain free of other metabolic deficiencies. Taken together, it was hypothesized that the *E. coli* mutants deficient in stringent response produced less catalase and therefore were more susceptible to bactericidal antibiotic selection compared to the wild type.

Two mutant strains of *E. coli* derived from *E. coli* K-12 were used to investigate the effect of stringent response: *rel*A single deletion and a double deletion of *rel*A and *spot*. Previous studies have shown that valine effectively inhibits the synthesis of isoleucine in *E. coli* K-12 (9). Valine and isoleucine biosynthesis utilizes the same enzymes, and both are regulated by end-product accumulation. High concentrations of valine have been shown to inhibit the condensing enzyme, which catalyzes the formation of an isoleucine precursor (10). We selected kanamycin (KAN), a bactericidal aminoglycoside that normally affects *E. coli*, to challenge all strains under stringent response and normal growth conditions (6).

Catalase levels were assayed after valine starvation via a hydrogen peroxide floating disk assay. Cell viability after overnight incubation with KAN was determined through killing and XTT assays to assess antibiotic tolerance in response to starvation. The results showed a greater increase in catalase production and tolerance to KAN under starvation conditions in BW25113 than the mutant strains, supporting the hypothesis.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *E. coli* K-12 BW25113 (WT), JW2755 (*Δrel*A*::kan)*, and SL11W447-4 (*Δrel*A*::kan/Δspo*T*::cam)* were obtained from the MICB 421 culture collection in the Microbiology and Immunology Department of the University of British Columbia (1, 11, 12). Both *Δrel*A and *Δrel*A/*Δspo*T mutants had resistance cassettes inserted, with flanking FLP recognition elements (1, 12). Cultures were grown in Luria-Bertani medium (in distilled water: 1% tryptone (BD 211921), 0.5% yeast extract (BD 210929), 1% NaCl (Fisher BP358-1) for liquid broth or with the addition of 1.5% agar (Invitrogen 30391-023) for plating) or M9 minimal salts medium supplemented with glycerol (in distilled water: 0.05% NaCl (Fisher BP358-1), 0.7% $Na_2HPO_4$ (Merck SX0720-1), 0.3% $KH_2PO_4$ (Merck PX1570-1), 0.1% $NH_4Cl$ (EMD AX1270-1), 0.02% $MgSO_4$ $7H_2O$ (Fisher M63-500), 0.2% glycerol (Fisher G31-1)). LB plates containing antibiotics were made, containing either 100 µg/ml ampicillin (AMP) (Sigma A-9518), 50 µg/ml KAN (Life 11815-024), or 25 µg/ml chloramphenicol (CAM) (Sigma C-0378). Specific growth conditions are listed in their respective experiments. All antibiotics and small aliquots of media were filter sterilized using a 45 µm filter.

**Plasmids.** pCP20 was obtained from the MICB 421 culture collection in the Microbiology and Immunology Department of the University of British Columbia. Extracted from *E. coli* BT340, the thermosensitive plasmid contains an $Amp^r$ marker and FLP recombinase helper (13). It was used to excise the $Kan^r$ gene in the *Δrel*A mutant, and both the $Kan^r$ and $Cam^r$ genes in the *Δrel*A/*Δspo*T mutant. Plasmid replication was prevented via incubation at 42°C.

**Preliminary antibiotic screening.** All strains were grown on LB agar with AMP, KAN, or CAM to screen for resistance phenotypes to confirm strain identity. Isolated colonies were selected from overnight cultures grown on LB plates at 30°C and inoculated onto LB plates containing antibiotics. Plates were then incubated overnight at 30°C and assessed for growth.

**Generation of competent cells.** Electrocompetent cells were required for the transformation of *Δrel*A and *Δrel*A/*Δspo*T mutants with pCP20, and preparations for both strains were identical. Competent cells were generated following an adapted manufacturer's protocol (14). Overnight cultures were used to inoculate 30 ml LB medium (1/30 dilution), then grown at 30°C. At $OD_{600}$ 0.5-0.7, cells were chilled on ice. 20 ml was transferred to a pre-chilled centrifuge tube and spun at 4000 ×g for 15 min at 4°C. The supernatant was discarded, and the pellet was washed twice with 20 ml 10% glycerol (Fisher G31-1) chilled on ice, once more with 1 ml 10% glycerol, and resuspended in 200 µl 10% glycerol.

**Electroporation of competent cells.** Transformed cells were generated following an adapted manufacturer's protocol (14). In a pre-chilled microfuge tube, 60 µl of competent cell suspension was mixed with 400 ng of the pCP20 plasmid DNA and incubated on ice for 1 min. After incubation, cells were transferred to a 0.2 cm electroporation cuvette (Bio-Rad) and electroporated at 2.5V using the Bio-Rad Micropulser™. The resulting suspension was incubated at 37 °C for 1 hr at 225 rpm in 1 ml of SOC medium (made up in distilled water: 2% tryptone (BD 211921), 0.5% yeast extract (BD 210929), 0.05% NaCl (Fisher BP358-1), 0.0186% KCl (Sigma P-9541), 0.24% $MgSO_4$ (Fisher M65-500), 0.36% glucose (Sigma G-5000)). Colonies were selected based on growth after plating on solid media containing 100 µg/ml AMP.

**Antibiotic resistance cassette excision by pCP20-mediated flippase.** $Kan^r$ cassettes in *Δrel*A and *Δrel*A/*Δspo*T mutants and the $Cam^r$ cassette in the *Δrel*A/*Δspo*T mutant were excised based on an existing protocol adapted from Datsenko (11) with the following modifications. The mutant strains were made electrocompetent, and then transformed with the pCP20 plasmid. After transformation, cells were plated on agar containing AMP and grown at 30°C to select for $Amp^r$ colonies. Ten colonies were subcultured onto non-selective LB plates and grown at 42°C overnight to inactivate the pCP20 plasmid. Temperature-treated cultures were streaked onto non-selective LB plates. Cultures were tested for AMP, KAN, and CAM resistance by restreaking individual colonies onto antibiotic plates. Successful removal of the antibiotic cassettes yielded colonies that were sensitive to all 3 antibiotics. These newly excised and antibiotic susceptible strains were named JKLL12W-1 (*Δrel*A, created from JW2755) and JKLL12W-2 (*Δrel*A/*Δspo*T, created from SL11W447-4).

**Minimal inhibitory concentration (MIC) assays.** Overnight cultures of WT, *Δrel*A and *Δrel*A/*Δspo*T strains were grown in M9 medium. Using MICs determined by Chenne *et al* (15), a 96-well plate (BD 353072 Microtest™ 96) was prepared with KAN. Wells A1-A9 contained M9 media as an uninhibited growth control, while wells B1-B9:F1-F9 contained M9 media with sufficient KAN for final concentrations of 8, 10, 12, 14, and 15 µg/ml respectively. Media and antibiotics (150 µl) were added per well along with 50 µl of cultures (columns 1-3: WT, columns 4-6: *Δrel*A, columns 7-9: *Δrel*A/*Δspo*T). The plates were incubated at 37°C overnight and assessed for visible bacterial growth. MICs of each strain were determined by the lowest antibiotic concentration which inhibited growth.

**Valine-induced starvation and split-stream culturing.** Cultures of WT (1/50 dilution), *Δrel*A (1/50 dilution) and *Δrel*A/*Δspo*T (1/20 dilution) strains were inoculated in 5 ml M9 media, and incubated overnight at 30°C and 225 RPM. Dilutions were chosen to achieve similar turbidity in all cultures after overnight incubation. Some overnight cultures (3 ml) were transferred to 7 ml M9 media and incubated for 2 hr at 30°C and 225 RPM. Some culture (5 ml) were kept in M9 ("unstarved condition"), while 4.5 ml of culture was supplemented with 0.5 ml of 1 mg/ml L-valine (Sigma V-0500) to induce isoleucine starvation. After 1.5 hr of incubation at 37°C and 225 RPM, the cultures were used for catalase and antibiotic tolerance assays.

**Specific catalase assay.** Catalase activity was measured by the time required for solution-soaked filter paper disks to float to the surface of a 1% hydrogen peroxide solution, based on the protocol used by Hsieh *et a*l and Chan *et al* (16, 17). A 1% (v/v) hydrogen peroxide solution was prepared from the 30% stock solution (VWR VW3742–1). 16 µl of test culture was absorbed onto 5 mm disks of 3 mm filter paper (#3 Whatman) for 10 s before being dropped into 50 ml of 1% (v/v) $H_2O_2$, which was approximately 3 cm of liquid height in a 100 ml beaker. Catalase activity was determined as the inverse of the time taken for the disk to float to the top. Disks that sank to the bottom or clung to the walls of the beaker were disregarded and the samples repeated. As well, turbidity measurements of the original culture were used to normalize the data so that it reflected specific catalase activity to account for differences in culture growth.

**Antibiotic tolerance assays.** A 96-well plate was prepared with KAN. A final concentration of 0, 3, 6, and 12 µg/ml of KAN was aliquoted respectively in columns 1-3, 4-6, 7-9, and 10-12. WT, *Δrel*A and *Δrel*A/*Δspo*T unstarved and starved cultures were

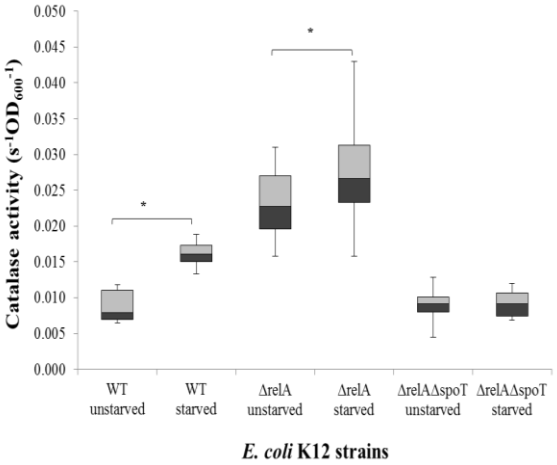


**FIG 1** Specific catalase activity under different nutrient conditions. Specific catalase data was collected from 4 independent trials. A t-test performed in SigmaPlot for each strain suggested significant differences in catalase production due to starvation for BW25113 and JKLL12W-1 as indicated by asterisks (P-values: BW25113:$2.49\times10^{-11}$, JKLL12W-1: 0.0297). JKLL12W-2 was not significantly different under starvation.

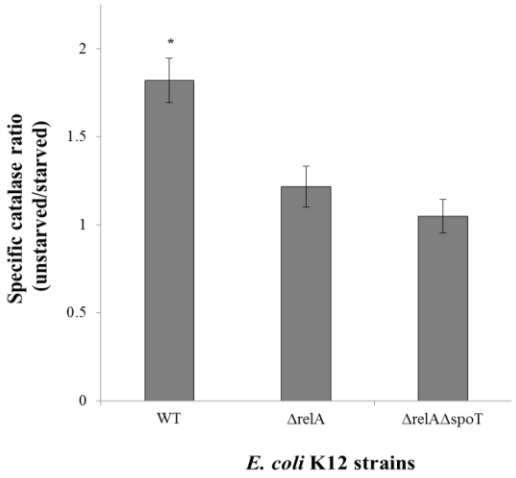


**FIG. 2. Specific catalase activity ratios of unstarved and starved cultures within each strain**. Data collected from 4 independent trials were used. The mean of specific catalase activity from all trials were used to calculate the ratio. Asterisk indicates significant inter-group difference. The error bars reflect standard error.

aliquoted into rows A-F, with each row being one strain. Each well contained 150 µl of M9 media and KAN, and 50 µl of culture. A negative control was aliquoted into wells G1-G3, containing only 200 µl of M9 media. Plates were incubated at 37°C overnight and growth was assessed by $OD_{600}$ turbidity measurements.

XTT/menadione assay mix was made from 12.5 XTT: 1 menadione (v/v) using stock solutions of 1 mg/ml XTT (Invitrogen X6493) dissolved in PBS (for 100 ml of 10x PBS: in distilled water, 1.236% $Na_2HPO_4$ (Merck SX0720-1), 0.180% $NaH_2PO_4$ (Sigma S9638), 8.5% NaCl (Fisher BP358-1)) and 1 mM menadione (reagent grade; used Nutritional Biochemicals Corp.) dissolved in acetone (reagent grade). XTT mix (50 µl) was aliquoted to each well and incubated for 1-3 hr at 37°C. Plates were read at 450 nm to quantify XTT reduction.

**Statistical analysis.** T-tests were performed to determine if the difference in catalase production among different strains and conditions was significant, while 1-way ANOVA was performed on the XTT and MIC data. SigmaPlot 12.5 and Microsoft Excel 2007 were used to analyze data.

## RESULTS

**Excision of antibiotic resistance markers.** A preliminary antibiotic screen confirmed the presence of resistance cassettes in JW2755 (Δ*rel*ACam$^r$) and SL11W447 (Δ*rel*A/Δ*spo*TCam$^r$/Kan$^r$/Amp$^r$) (1, 12). The pKD46 plasmid containing Amp$^r$ was not removed from the SL11W447, and incubation at 42°C successfully inactivated plasmid replication and restored AMP susceptibility. JW2755 and AMP-sensitive SL11W447 were successfully transformed with the flippase-containing pCP20 gene, as confirmed by the acquisition of AMP resistance. Colonies from LB plates after 42°C were subjected to antibiotic screening, and while colonies grew on the positive control plate, no colonies grew on antibiotics. Thus, antibiotic resistance cassettes were successfully inactivated. Two new strains were produced, with their respective resistance markers removed: JKLL12W-1 (Δ*rel*A) and JKLL12W-2 (Δ*rel*A/Δ*spo*T).

**Catalase production upon starvation decreased in mutants**. Comparisons among the 3 strains under different nutrient conditions were performed. Catalase activity was measured by the floating disk assay and calculated as the inverse of the time it took for disks to float, normalized by the turbidity. Figure 1 showed that while WT and the Δ*rel*A mutant had increased catalase under starvation (103% and 16% increase respectively), the Δ*rel*A/Δ*spo*T mutant did not show a noticeable increase in catalase. The result was expected because Δ*rel*A/Δ*spo*T should be incapable of inducing stringent response, due to the absence of *rel*A and *spo*T. However, it was unexpected that Δ*rel*A was able to produce more catalase than WT, as the mutant would be expected to produce less catalase overall, correlating to a deficient stringent response pathway because of the absence of *rel*A. Figure 2 showed that while Δ*rel*A produced more catalase than WT in Figure 1, WT had a significantly greater increase in catalase production under starvation, when normalized to unstarved production, compared to the other two strains (49.5% to Δ*rel*A and 73.5% to Δ*rel*A/Δ*spo*T). The result supported the hypothesis, because the inactivation of *rel*A and *spo*T should have caused deficient stringent response, which in turn decreased catalase production under starvation.

**Viability and growth of mutants decreased upon starvation**. A preliminary experiment indicated that 12 µg/ml of KAN was sufficient to noticeably inhibit the growth of all 3 strains (data not shown). A series of KAN concentrations, ranging from 3-12 µg/ml, was used for the XTT and killing assay to correlate trends in viability to increased antibiotic concentration. A trend of decreased viability with increased concentration of KAN was observed for both the Δ*rel*A and Δ*rel*A/Δ*spo*T strains grown under unstarved and starved conditions (Fig. 3A).

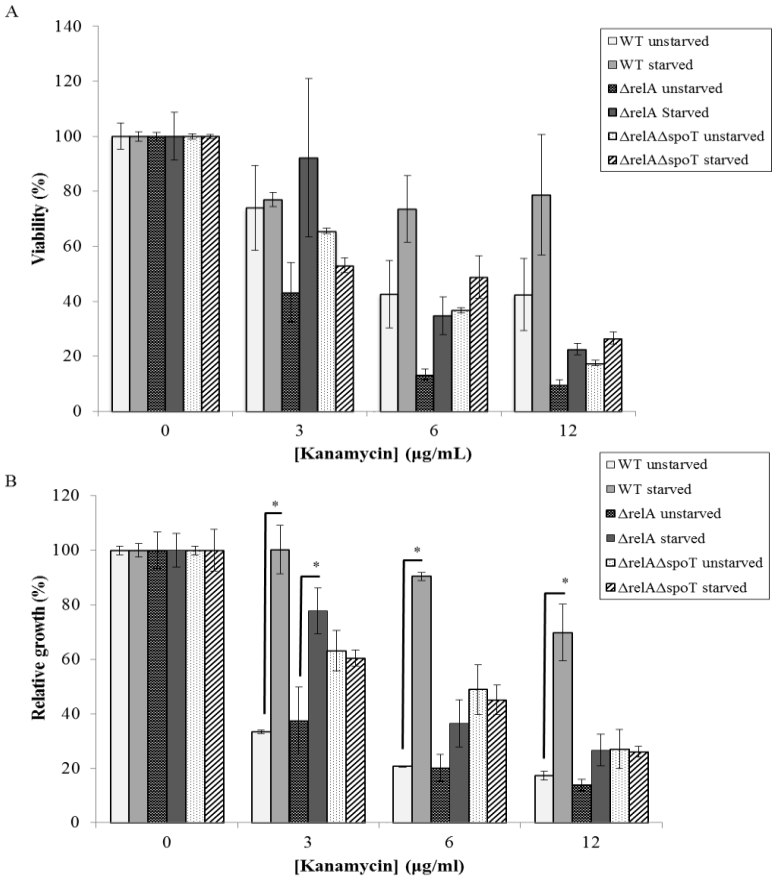


**FIG 3** (A) Percent viability shows XTT results and (B) relative growth after overnight selection with kanamycin shows results from the killing assay. Mean viability from 2 independent trials was used. Standard error is expressed as error bars. Intra-group normalization was performed with each strain normalized against its respective zero antibiotics control. Asterisks indicate significant differences (P-value < 0.02).

However, the unstarved and starved WT strain levelled off at 6 µg/ml and 3 µg/ml KAN respectively. At 12 µg/ml KAN, the starved WT culture contained 3.5× more viable cells than the starved Δ*rel*A culture, and 3× more viable cells than the starved Δ*rel*A/Δ*spo*T culture. Higher tolerance to KAN observed in the WT compared to the Δ*rel*A and Δ*rel*A/Δ*spo*T was expected, since WT has a fully functional stringent response pathway that mediates tolerance to KAN. It was predicted that the Δ*rel*A/Δ*spo*T mutant lacking stringent response would be more susceptible to KAN under starved conditions than the Δ*rel*A single mutant. The prediction was not supported as there was no significant difference in viability between these 2 strains at any concentration of KAN. As variability in the XTT assay was extremely high between replicates, all conclusions were derived from trends within the data.

With the killing assay, a trend of decreased viability with increased concentrations of KAN was observed for all 3 strains under both starved and unstarved conditions (Fig. 3B). Significant growth advantage of the WT strain under starved conditions compared to unstarved was observed for all concentrations of KAN, while growth of the starved Δ*rel*A mutant was only significantly greater than unstarved at 3 µg/ml KAN (Fig. 3B). No significant difference in growth was found between starved and unstarved Δ*rel*A/Δ*spo*T cultures. WT was better able to increase tolerance to KAN under starvation than both mutant strains. At 3 µg/ml KAN,WT grew 1.3× more than Δ*rel*A and 1.7× more than Δ*rel*A/Δ*spo*T to KAN. However, the difference between the two mutant strains was less obvious as KAN concentrations increased. At 12 µg/ml KAN, the starved WT culture grew 2.6× more than the starved Δ*rel*A culture and 2.7× more than the starved Δ*rel*A/Δ*spo*T culture.

## DISCUSSION

Activation of the stringent response in *E. coli* through the addition of excess valine was shown to increase resistance to a wide variety of antibiotics, including KAN (18). However, the cause for this resistance is unclear. This study proposed that during stringent response, induction of *kat*E increases catalase production, which acts to combat the reactive oxygen species produced by the antibiotics. The results from this investigation showed a correlation between catalase activity and increased antibiotic tolerance in *E. coli* under stringent response.

A 103% increase in catalase activity in WT under starvation showed that catalase production was upregulated compared to unstarved cultures (Figure 1). The increase in catalase production in WT was consistent with the results obtained from *P. aeruginosa* in previous research and expected as stringent response upregulates many genes essential for survival, including those for oxidative stress control. Relative to WT increase in catalase production, Δ*rel*A and Δ*rel*A/Δ*spo*T cultures were less efficient at inducing catalase production under starvation conditions, producing a 16% increase in activity in Δ*rel*A and no increase in Δ*rel*A/Δ*spo*T. The reduced ability of *rel*A mutants to upregulate catalase activity could be due to their limited ability to synthesize (p)ppGpp and consequently little to no activation of stringent response. RelA and SpoT both possess (p)ppGpp synthase capabilities; however, the (p)ppGpp synthase activity of SpoT is weaker than that of RelA (4). Since *rel*A mutants can only rely on *spo*T for (p)ppGpp production, the effects of stringent response will be less pronounced, consistent with the observations for Δ*rel*A/Δ*spo*T. The double deletion mutant completely lacks the ability to synthesize (p)ppGpp and relies on constitutive *kat*G for catalase production.

However, the trend for specific catalase activity among the strains was not as clear. The Δ*rel*A strain had a higher baseline catalase activity compared to the WT and Δ*rel*A/Δ*spo*T strains. This could be a result of a discrepancy in constitutive catalase expression in these different strains. In addition, growth properties also influence the expression of *kat*G under normal conditions. *kat*G expression is regulated by *oxy*R in *E. coli*, which in turn is up-regulated by endogenous $H_2O_2$ production correlating to proliferation (19). The growth properties of different strains could be elucidated by growth curves. As well, the difference in baseline catalase production could be attributed to variability in growth and starvation conditions. The variability among trials suggests that these conditions need to be better controlled for consistency in the future.

In addition, the floating disk assay is highly variable as it relies on a few assumptions: the rate of hydrogen peroxide entering the cells is consistent, the loss of oxygen bubbles from the top surface as catalase breaks down hydrogen peroxide is consistent, equivalent biomass is transferred to each filter paper, and the rise of filter paper is consistent among all replicates. The difficulty in controlling these factors contributes to the observed variability. Although most results were statistically significant in our experiment, more accurate methods of measuring catalase should be explored to minimize variability.

In comparison to WT, Δ*rel*A and Δ*rel*A/Δ*spo*T strains were expected to be more sensitive to KAN under starvation. This was consistent with the results seen in Figure 3A. The viability of all strains decreased with increasing KAN concentration, but this decrease was less prominent in the WT strain under starved conditions. This also correlated to the significant increase in growth for the starved versus unstarved WT cultures (Fig. 3B). The WT strain was more tolerant to KAN at all concentrations when under starvation. The trend of increased tolerance across all strains correlated to the increased activity of catalase seen under the same conditions.

The XTT viability assay showed greater inconsistency among the replicates, as well as a less obvious trend among the conditions, which could be due to growth differences, as overgrown cultures contain more cells but may not be sufficiently metabolically active to reduce XTT efficiently. The optimum XTT digest time was around 1.0-1.5 hours, which is 2× shorter than expected, suggesting the presence of a large amount of bacteria in all wells. This was further confirmed by the high $OD_{600}$ readings in some wells (0.3-0.5, data not shown). It is possible that the XTT reagent was not in excess of the bacterial culture, and the product became saturated in many samples. Saturation will decrease observable differences in absorbance across samples, producing inconclusive results. The XTT assay results suggested that it is important to regulate and control the amount of seeded bacteria. Alternatively, increasing the XTT concentration could also increase the observable difference. Variability among replicates can also be attributed to the amount of time after addition of KAN to the cultures at which the XTT assay is performed. Furthermore, aminoglycoside antibiotics, such as KAN, cause a free radical burst upon initial exposure to bacteria. These radicals have been shown to reduce XTT (20). Therefore, incubation with XTT too soon after this radical burst may lead to increased turnover of the XTT, and false readings of viability. The false readings of viability was seen when plates were assayed with XTT after 2 hr incubation with KAN (data not shown).

Taken together, a correlation between a high catalase production due to stringent response and KAN tolerance could be implicated, which supports the initial hypothesis and suggests a possible pathway to antibiotic resistance in *E. coli*. Compared to the WT, the Δ*rel*A and Δ*rel*A/Δ*spo*T strains, relative to unstarved conditions, produced less catalase and were less tolerant to KAN after starvation. Specifically, the double mutant lacking both RelA and SpoT was found to be the least tolerant to KAN upon starvation, reflecting Spot's ability to elicit stringent response in absence of RelA. The increased KAN susceptibility upon SpoT elimination suggested a positive correlation between the functionality of the stringent response pathway and antibiotic tolerance in *E. coli*. However, the increased catalase production in the Δ*rel*A strain compared to the WT strain is anomalous, and the result needs to be investigated further. Further experiments are needed to explore the resistance mechanism and directly attribute KAN tolerance to increased catalase production.

## FUTURE DIRECTIONS

Stringent response induces tolerance to a broad range of bactericidal antibiotics with different mechanisms of action. However, due to time limitations, KAN was the only antibiotic tested. The three strains of *E. coli* should be exposed to other classes of antibiotics in order to

determine if oxidative stress control underlies the tolerance observed. Some possible candidates include β-lactams and fluoroquinolones.

The floating disk assay was chosen based on available resources and previous success with the technique (16,17). The accuracy of the catalase quantification assay can be improved by measuring hydrogen peroxide breakdown via spectrophotometry or colorimetry. Measurement via spectrophotometry could reduce some of the variability related to observation of filter papers in hydrogen peroxide. Alternatively, a colorimetric assay using potassium dichromate and acetic acid is available, as well as various commercially available kits. Both methods eliminate the rate of hydrogen peroxide uptake by testing catalase levels in cell lysates, as well as the inconsistencies from the use of filter papers (21).

The XTT assay also requires optimization in order to yield consistent meaningful results. Incubation times and reaction volumes need to be re-evaluated. We assumed the concentration of antibiotics in the wells would not significantly contribute to the reduction of XTT. However, antibiotics cause a free radical burst that can contribute to the chromogenic substrate produced and higher spectrophotometer readings. In addition, incubation times need to be optimized based on $OD_{600}$ measurements; the time required for the XTT reaction to develop can vary from 1-2 hr depending on the turbidity.

While this study had implied a correlation between KAN tolerance and catalase production, the direct effect was not studied. The *rel*A and *spo*T mutations decrease (p)ppGpp production, which is involved in the regulation of many downstream cascades (4). It is therefore difficult to attribute the increase in catalase production solely to KAN. More direct support is needed for the role of oxidative stress proteins in antibiotic tolerance and could be generated with a strain of *E. coli* deficient in both *kat*G and *kat*E. When compared to wild-type *E. coli*, catalase knockout mutants are predicted to have lower antibiotic tolerance. In addition, to elucidate the mechanism of action, $H_2O_2$ breakdown could be measured by absorbance at 240 nm and compared between the *kat*G and *kat*E mutants to support the hypothesis that $H_2O_2$ neutralization is a primary cause of bactericidal antibiotic tolerance. However, since eliminating *kat* genes does not affect the stringent response pathway, *kat* mutants cannot be used to test the impact of stringent response directly but only to confirm that catalase has an impact on antibiotic tolerance.

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