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Stringent Response Triggered by Valine-Induced Amino Acid Starvation Does Not Increase Antibiotic Tolerance in *Escherichia coli* Cultures Grown at Low Cell Density

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The stringent response is a survival mechanism in bacteria that is activated by various environmental stressors, including nutrient limitation such as amino acid starvation. The hallmarks of the stringent response include slowed growth and decreased synthesis of translational machinery (tRNA and rRNA) in favour of increased expression of stress response genes. In Escherichia coli, these include genes involved in combating oxidative stress, such as catalase. Treatment of E. coli K-12 with excess valine is a well-established method of inducing isoleucine starvation to trigger the stringent response and has been previously correlated with increased tolerance to beta-lactams and aminoglycosides. In this study, the effect of the stringent response on antibiotic tolerance to ampicillin, kanamycin and ciprofloxacin were investigated using E. coli K-12 wild type (WG1), relA mutant (AT-2) and relA/spoT double mutant (AB301). Induction of the stringent response was confirmed using a floating disc assay to measure catalase activity and a Bradford assay to measure total protein. Short term and long term antibiotic resistance were assessed through the generation of growth curves following incubation of replete and starved cultures with sub-minimum inhibitory concentrations of antibiotic and minimum inhibitory concentration (MIC) assays. The results showed that under starvation conditions, catalase production was higher in wild type compared to the mutant strains, indicating that the stringent response had been induced, while total protein results were highly variable. Despite the increase in catalase production under starvation, no differences in MIC or growth were observed across replete and amino acid starved cultures when challenged with any of the three antibiotics, indicating that valine-induced isoleucine starvation did not have an effect on ampicillin, kanamycin or ciprofloxacin tolerance.

Bacteria undergo a dramatic physiological reprogramming known as the stringent response (SR) under environmental stressors such as amino acid starvation or heat shock. This mechanism aids in the survival of the bacterium by downregulating the expression of genes required for tRNA synthesis, rRNA synthesis and growth, while upregulating those required for stress response (1, 2). The alarmones guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and guanosine 5'-triphosphate 3'-diphosphate (pppGpp), collectively referred to as (p)ppGpp, orchestrate this widespread transcriptional switch in Escherichia coli by accumulating inside the cell in response to environmental stressors and directly binding to RNA polymerase (3, 4). Cellular levels of (p)ppGpp are regulated by two enzymes, RelA and SpoT, both of which respond to different stressors. SpoT senses limitations in carbon, fatty acids, phosphate and iron, possesses weak (p)ppGpp synthetase activity and serves as the sole (p)ppGpp hydrolase, whereas RelA is capable of (p)ppGpp synthesis only in response to either amino acid starvation or heat shock (5). In the specific case of amino acid limitation, the accumulation of deaminoacylated tRNA, a consequence of amino acid starvation, results in a deaminoacylated tRNA entering the ribosomal acceptor site, causing ribosomal stalling. RelA associates with the stalled ribosome, detects the deaminoacylated tRNA, and begins synthesizing (p)ppGpp in response (2, 5). In the presence of RelA, the (p)ppGpp hydrolysis function of SpoT is essential for balancing cellular concentrations of (p)ppGpp, but the molecular details on how SpoT mediates this hydrolysis is still unknown, as efforts to purify recombinant SpoT from E. coli for functional analysis have yet to be successful (5).

Besides protecting bacteria from the stressor, these changes in cell physiology and gene expression in response to stress are believed to impact bacterial tolerance to antibiotics. SR-induced tolerance to antibiotics is believed to not only arise indirectly as a result of stress-induced growth arrest, as antibiotics typically target processes that only occur in growing cells such as peptidoglycan, protein or DNA synthesis, but also directly as a result of stress-induced changes to antibiotic targets, recruitment of antimicrobial resistance factors such as multidrug efflux pumps (6) and the recruitment of oxidative stress control enzymes (7). Previous studies indicate that major bactericidal antibiotics, such as beta-lactams, aminoglycosides and fluoroquinolones, mediate cell death not only through the binding of their targets, but also through the generation of reactive oxygen species (ROS) such as superoxide ($O2\cdot$), hydrogen peroxide ($H2O2$) and hydroxyl radicals ($OH\cdot$) (8, 9). The role of oxidative stress control in antibiotic tolerance is further shown in a recent study that observed a correlation between kanamycin tolerance and increased catalase activity in E. coli following valine-induced isoleucine starvation (10). In E. coli, two catalases, hydroperoxidase I (encoded by katG) and hydroperoxidase II (encoded by katE), enables the bacterium to resist oxidative stress by catalyzing the reaction $H2O2 \rightarrow H2O + ½\ O2$ (11).

Excess valine induces isoleucine starvation by feedback inhibition, as the biosynthesis of both amino acids occur in

parallel and involve the same enzymes (12). Valine-induced isoleucine starvation has been previously shown to activate the SR in E. coli K-12 and has been correlated with increased tolerance to penicillin and kanamycin following two hour and overnight resistance studies, respectively (3, 10). However, a similar study that investigated the effect of SR activation by either isoleucine starvation or sublethal kanamycin treatment did not observe a difference in ampicillin tolerance across control and starved/sub-inhibitory kanamycin-treated wild type and relA mutant strains (13). This short term resistance study involved growth rate measurements across a one hour interval (13).

Most of the resistance studies in E. coli so far have tested antibiotics from either the beta-lactams or aminoglycosides classes. In this experiment, we aim to examine whether the effect of valine-induced isoleucine starvation activated SR is a general response that extends to fluoroquinolones, such as ciprofloxacin, which acts on DNA gyrase and topoisomerases to inhibit DNA replication (14). Four E. coli K-12 strains were used for this experiment: wild type (WG1), relA mutant (AT-2), relA/spoT double mutant (AB301) and katE/katG double mutant (PN11W-4b). The relA and relA/spoT mutant strains were used to investigate the effects of the SR. The katE/katG mutant was used as a negative control in the catalase assay only. Relative catalase activity and total protein were compared in replete and valine-induced starved cultures to aid in the characterization of the SR and to provide confidence that the SR had been activated. To assess for relative differences in short-term and long-term antibiotic tolerance following starvation, growth curves were generated and minimum inhibitory concentration (MIC) assays were performed on replete and starved cultures challenged with ampicillin, kanamycin or ciprofloxacin.

MATERIALS AND METHODS

Bacterial strains and preparation of overnight cultures. E. coli strains WG1 (K-12 WT), AT-2 (relA1), AB301 (relA/spoT) and PN11W-4b (katE/katG) were obtained from the MICB 421 culture stock collection in the Department of Microbiology and Immunology at the University of British Columbia. To prepare overnight cultures, an isolated colony of each strain was inoculated in 10 mL of prepared M9 minimal media and incubated overnight at 200 rpm and 37oC.

Preparation of modified M9 minimal salt media. The modified M9 minimal media was prepared as described by Chiang et al. with some modifications (13). The following three solutions with the given final concentrations (in combined media) were autoclaved separately: solution 1 was prepared with 0.5 mg/mL NaCl (VWR 0241-1KG), 7.0 mg/mL Na2HPO4 (EM SX0720-5), 3.0 mg/mL KH2PO4 (EM PX1565-1) and 1.0 mg/mL NH4Cl (BDH B10017); solution 2 was prepared with 0.2 mg/mL MgSO4 · 7H2O (Fisher M-65) and 3.5 mg/mL glycerol; and solution 3 was prepared with 2.0 mg/mL Bacto casamino acid (BD 223120), 0.05 mg/mL L-glutamine (Sigma G-5763), 0.05 mg/mL L-asparagine (Sigma A-0884), and 0.01 mg/mL tryptophan (Sigma T-0129). The following two solutions with the given final concentrations (in combined media) were filter-sterilized: solution 4 was prepared with 1 µg/mL biotin solution (Invitrogen 19524-016) and 1 µg/mL thiamine solution (Sigma T-4625) and solution 5 was prepared with 0.3 mL of 1 M CaCl2 per 1 L of medium. These five solutions were combined and 5 N NaOH (EMD SX-0590-1) was added to adjust the pH to 7.2 units.

Preliminary antibiotic screening. Isolated colonies from overnight cultures of each WT, relA mutant and relA/spoT double mutant strains streaked on Luria Broth (LB) agar plates were selected and grown on antibiotic containing LB plates. All strains were grown on 10 µg/mL ciprofloxacin-LB plates (Sigma 17850), 30 µg/mL ampicillin-LB plates (Sigma A-9518) and 30 µg/mL kanamycin-LB plates (Life 11815-024) to confirm that the strains are not resistant to the antibiotics of interest. The plates were incubated for 3 days at room temperature.

Preparation of unstarved and starved cultures. Overnight culture was used to inoculate fresh M9 minimal media to obtain a final volume of 45 mL and a final OD600nm of 0.15 units. Cultures were then incubated at 37oC for one hour on a shaker at 200 rpm. After the incubation, OD600nm reading of each culture was taken using a Pharmacia Biochrom Ultrospec 3000 UV/ Visible Spectrophotometer and normalized through dilution to achieve the same OD600nm across each strain. Next, each culture was split into 20 mL of unstarved culture and 19 mL of starved culture. For the starved cultures, 1 mL of 10 mg/mL L-valine (Sigma V-0500) was added so that the final volume was 20 mL and the final L-valine concentration was 0.5 mg/mL L-valine. The prepared unstarved and starved cultures were then incubated at 37oC for 3 hours.

Catalase assay. The floating disc assay adapted from Hsieh et al. (15) was used to evaluate catalase activity by measuring the time required for the culture-soaked filter paper disks dropped into 1% hydrogen peroxide solution to float to the surface. Cultures of starved and unstarved WT, relA and relA/spoT double mutant were measured using spectrophotometer at 600 nm before the start of the assay. 50 mL of 1% (v/v) H2O2 solution, prepared from a 30% (v/v) H2O2 solution (BDH 7690-1), was added to a 100 mL beaker. 20 µL of culture was pipetted onto 3 mm Whatman #3 filter paper and the disks were allowed to soak for 10 seconds before being placed in the 1% (v/v) H2O2 solution. Catalase activity was calculated as the inverse time required for the disks to float to the surface of the 1% H2O2 solution, in a beaker with a 2.75 cm liquid height, multiplied by the inverse OD600nm reading of the culture being tested. Disks that clung to the side of the beaker were disregarded and each culture was measured four times.

Preparation of cell lysates. Using the unstarved and starved cultures prepared, the preparation of cell lysate was conducted. 10 mL of overnight culture was centrifuged at 7500 x g at 4oC for 5 minutes using a J2-21 centrifuge (Beckman). The resulting pellet was extracted, washed with 10 mL cold Tris buffer (25 mM Tris-HCl, pH 8) and centrifuged at 7500 x g, 4oC for 5 minutes. Resuspension in 1 mL Tris buffer (25 mM Tris-HCl, pH 8) was performed, followed by cell disruption using the FastPrep-24 Cell Disruptor (Misonix) for 30 seconds on setting 6. At the end, the cells were centrifuged at 14000 x g for 1 minute at room temperature using an Eppendorf 5415D Microcentrifuge. The supernatant, which contains proteins, was collected.

Total protein concentration assay (Bradford assay). Bradford assay was performed according to the manual provided by Bio-Rad (16). First, Bio-Rad Dye Reagent Concentrate (Bio-Rad 5000006) was diluted to a working concentration of 1x and filtered using a Whatman #1 filter. In order to construct a standard curve, a 1 mg/mL bovine serum albumin (BSA, Sigma A-4503) stock solution was prepared. Using the stock solution, ½ serial dilutions with cold Tris buffer (25 mM Tris-HCl, pH 8) were performed to obtain BSA concentrations ranging from 1 mg/mL to 0.00625 mg/mL. After that, 40 µL of each cell lysate and each BSA standard solution was incubated with 800 µL of dye solution in a microcentrifuge tube for a minimum of 5 minutes at room temperature. In a flat bottom 96-well microplate, 210 µL of each lysate and BSA standard solution was added to each well. Three replicates were assayed and 40 µL of Tris-Buffer that had been incubated with the dye reagent served as the blank. At the end, absorbance was read at 595 nm using a BioTek Micro-volume Plate Reader and a standard curve was constructed to allow for the determination of the total protein concentration of each cell lysate.

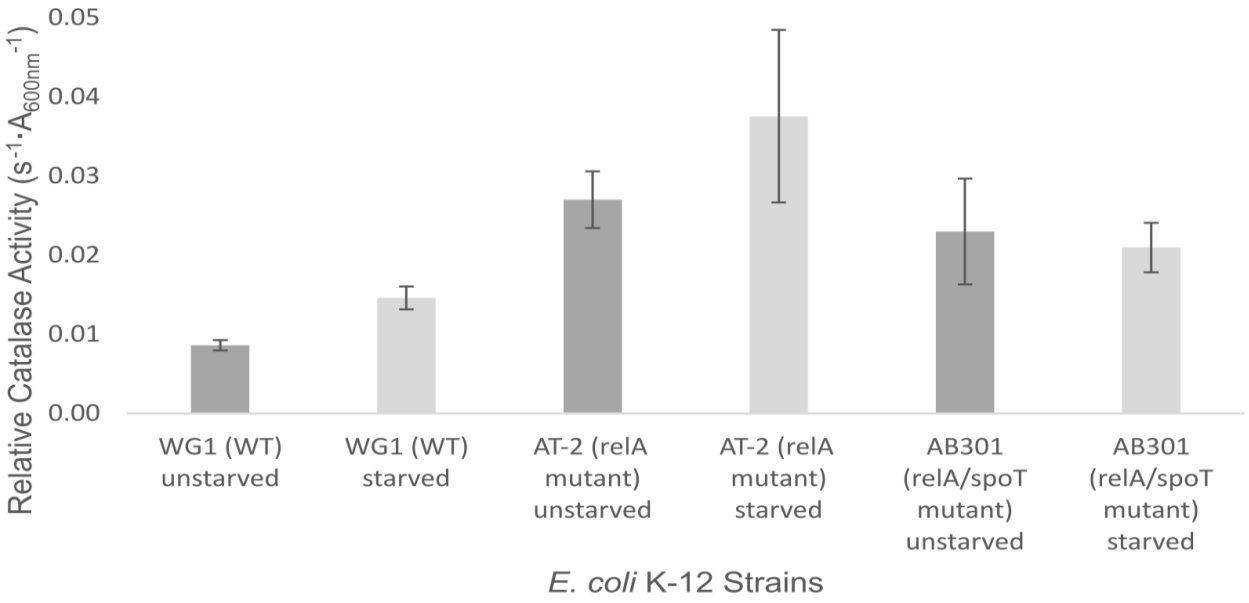

FIG 1. **Catalase activity of *E. coli* strains WG1 (WT), AT-2 *(relA*), AB301 (*relA/spoT*) and PN11W-4b (*katE/katG*) under control and starvation conditions.** Error bars represent 95% confidence intervals (n = 1, 4 replicates per culture).

Minimal Inhibitory Concentration assay. 10 mg/mL ciprofloxacin stock solution in 0.1 N HCl, 50 mg/mL ampicillin stock solution in distilled water, and 50 mg/mL kanamycin stock solution in distilled water were prepared and sterilized using 0.22-µm filters. Six sterile clear flat bottom 96-well plates were prepared by adding 100 µL of M9 media into each well. A final concentration of 0.5 µg/mL ciprofloxacin and 5 µg/mL of ampicillin and kanamycin were added and 1/2 serial dilutions were performed. 5 µL of 4.5 x 104 cells/mL of one of the starved and unstarved WT, relA and relA/spoT cultures were added to the wells. Negative controls contained M9 media alone and positive controls contained bacteria in the absence of antibiotics. The six plates were incubated overnight at 37oC and the turbidity was read the next day using a BioTek Micro-volume Plate Reader. Using the OD600nm measurement, growth inhibition in each well was determined by finding the well that displayed an increase in absorbance of at least two times that of the previous well and taking the antibiotic concentration of that previous well as the MIC concentration. In the rare case where there was no minimum two times difference in absorbance across wells in a row for a given sample, the most conservative antibiotic concentration (i.e. lower antibiotic concentration) with an absorbance closest to the negative control was selected as the MIC. In the case where the MIC of replicates of the same sample for a given antibiotic were not identical, the more conservative antibiotic concentration was determined as the MIC.

Growth Curve assay. Prior to the growth curve assay, the cultures were incubated at 37oC on a shaking platform at 200 rpm. After turbidity was measured, each culture was diluted to a final cell concentration of 0.015 OD600nm units in a final volume of 2 mL. Modified M9 minimal media was used as the diluent and an appropriate volume of 0.5 mg/mL L-valine was added to the starved cultures to induce amino acid starvation. Concentrations of ampicillin, kanamycin and ciprofloxacin used were the sub-MIC for the WT E.coli strain determined from the MIC assay; 1.25 µg/mL for ampicillin, 2.5 µg/mL for kanamycin and 3.91 ng/mL for ciprofloxacin. In a 96-well microplate, 100 µL of each culture was incubated with 100 µL of each antibiotic solution for 3 hours at 37oC. As a negative control, 100 µL of each culture was incubated with 100 µL of M9 media instead of an antibiotic solution. During the incubation period, OD600nm readings were taken at 30-min intervals using a BioTek Micro-volume Plate Reader. Turbidity measurements over time were used to construct a growth curve for each culture.

RESULTS

Catalase production under starvation conditions increased in WT and decreased in relA mutant in E. coli.

To determine if the SR had been activated under the starvation conditions, catalase activity was measured using a floating disc assay. Catalase activity was calculated as the inverse time required for the discs to float to the surface of a 1% H2O2 solution. This value was multiplied by the inverse OD600nm reading of the culture being tested to control for differences in turbidity across cultures. To control for false positives, katE/katG mutant cultures and M9 media served as the negative controls; these yielded zero catalase activity as expected since the strain is missing two catalase genes. Comparisons were made between starvation and control cultures of each strain. As expected, WT showed a significant increase in catalase activity under starvation compared to the mutant strains, as indicated by the non-overlapping 95% confidence intervals shown in Fig. 1. This was expected since the WT strains contain both genes involved in activating the SR. Also, as expected, the relA mutant under starvation exhibited higher catalase activity than the relA mutant unstarved control. However, the relA mutant showed higher catalase activity compared to WT. We would expect the relA mutant to have less catalase activity compared to WT due to the absence of relA. While the relA mutant strain produced more catalase than WT (Fig. 1), we show that WT had a greater increase in catalase production (1.7x higher;) compared to the other two strains (1.4x higher and a 0.088-fold decrease) after normalization to unstarved production. While the relA mutant induced a similar amount of catalase production under starvation as WT (1.4x versus 1.7x), the difference was not statistically significant. Indicated in Fig. 2, the relA/spoT mutant under starvation yielded a minor, non-significant difference in catalase

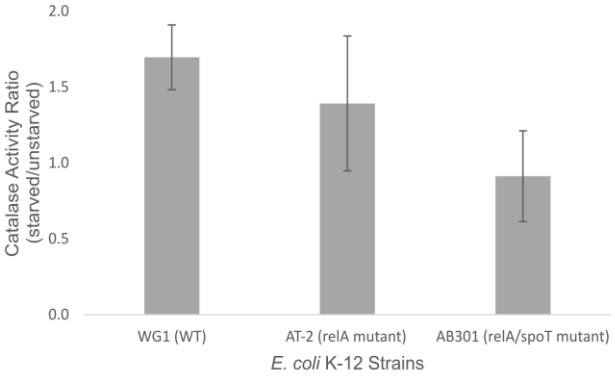

FIG 2. **Catalase activity ratios of starved to unstarved cultures within each strain**. n = 1, 4 replicates per culture. The mean catalase activity from all replicates were used to calculate the ratios. Errors bars represent 95% confidence intervals (n = 1, 4 replicates per culture).

activity, as it is missing both genes involved in SR induction. The result matches expectations as the absence of relA and/or spoT should result in a deficient SR, resulting in the decreased induction of catalase production under starvation.

Total protein concentration measurement is unreliable as an indicator of stringent response in E. coli. In order to test if the starvation condition (3 hour incubation in the presence of 0.5 mg/mL valine) could successfully induce the SR in the WT strain, cell lysates were prepared using a FastPrep Cell Disrupter and the Bradford assay was performed to measure the total protein concentration. We expected that if the starvation condition was inducing the SR, a significant reduction in total protein concentration would be observed in the WT and relA mutant cultures under starvation. The total protein concentrations measured in each strain and in each condition are shown in Fig. 3. We obtained a high variation across two trials; only the results from the relA mutant were consistent. In line with our expectations, decreased protein concentration was observed in the relA mutant under starvation. SpoT enzyme alone was sufficient to result in the reduction of protein concentration, a proposed characteristic of the SR in this strain. Meanwhile, for the other two strains, starvation did not result in any difference in total protein concentration when compared to the control condition. We obtained a large standard error of the mean, especially for WT and the relA/spoT mutant under starvation. Based on the inconsistency of these results, we could not conclude whether the starvation condition used had activated the SR in the WT strain. Total protein concentration assay was therefore not used to characterize the level of SR.

Stringent response induced under valine starvation does not confer long-term ciprofloxacin, ampicillin, and kanamycin tolerance in E. coli. In order to test if the SR leads to increased long-term ciprofloxacin, ampicillin, and kanamycin tolerance, we used the MIC assay (Table 1). Wells containing media served as a negative control, whereas wells containing culture in the absence of antibiotics served as a positive control. As shown in Table 1, there was no difference in MIC within the same strain under the starvation and control conditions, for any of the three antibiotics tested. This trend was confirmed with another trial that also revealed no difference in MIC for the three antibiotics under starvation and control conditions when WT E. coli was tested (Supplementary Table 1). These data suggest that SR activated by valine-induced amino acid starvation has no effect on long term tolerance to ciprofloxacin, ampicillin or kanamycin in E. coli.

Short-term adaptive resistance to antibiotics was not observed in starved cells. To test for short-term adaptive resistance to antibiotics in WT and relA E.coli undergoing SR, we constructed growth curves to compare the growth rates of the starved and unstarved cultures in the presence or absence of an antibiotic. The cultures were incubated with the sub-MIC of each antibiotic (ampicillin, kanamycin and ciprofloxacin) for 3 hours and the OD600nm of each culture was measured in 30-minute intervals. We defined the sub-MIC to be half of the MIC

for each strain (Table 1). Growth curves were constructed using turbidity measurements collected over time. Two trials were performed and similar results were observed across the two trials. The results from one trial are shown in Fig. 4. In the absence of an antibiotic, WT under SR had a similar growth rate compared to the control. As well, no differences in growth rate between starved and unstarved cultures of the relA and relA/spoT strains were observed. When sub-MIC of ampicillin, kanamycin, or ciprofloxacin was added, the resulting growth curves of the starved and unstarved cultures of each strain were similar (Fig. 4). As a higher growth rate was not observed in the starved WT and relA cultures in the presence of antibiotics, we conclude that there may not be short-term adaptive resistance to antibiotics in cells undergoing stringent response.

***E. coli* Culture**	**MIC (μg/mL)**		
	Ciprofloxacin	**Ampicillin**	**Kanamycin**
WT unstarved	0.0078	2.5	5
WT starved	0.0078	2.5	5
*rel*A unstarved	0.0039	5	2.5
*rel*A starved	0.0039	5	2.5
*rel*A/*spo*T unstarved	0.0039	2.5	2.5
*rel*A/*spo*T starved	0.0039	2.5	2.5

DISCUSSION

Table 1. Minimum inhibitory concentration of ciprofloxacin, ampicillin, and kanamycin on *E. coli* WG1 (WT), AT-2 (*rel*A), AB301 (*relA/spoT*) cultures under starved and unstarved conditions (n = 1).

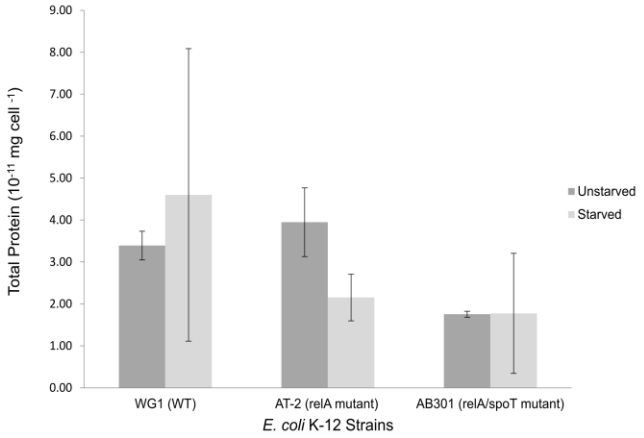

FIG 3. **The mean total protein (mg) per *E. coli* cell obtained from two independent trials.** Strains were WG1 (WT), AT-2 *(relA*), AB301 (*relA/spoT*) under control and valine-induced isoleucine starved conditions. Cultures were prepared by diluting overnight cultures to ~0.15 A_{600nm} in M9 minimal media, incubating for 1 hour at 37ºC, splitting each culture and having 0.5 mg/mL L-Valine in starved cultures. All cultures were incubated for 3 hours at 37ºC and then stored in 4ºC fridge before protein purification using Fastprep. Tris-HCl (25mM, pH 8) was the diluent that make the final volume to 1 mL. Bradford assay was performed to obtain the protein concentration of each cell lysate. Total protein of each culture was calculated by dividing total protein extracted (protein concentration x final volume obtained) with total cell number (10 mL of culture used x OD_{600nm} reading x OD factor 1E9). Error bars represent standard error of the mean.

Previous studies have shown a correlation between SR and antibiotic tolerance (6, 10, 13). Prior to the performance of the MIC and growth curve assays, to ensure that our starvation condition was capable of activating the SR in WT E. coli, we determined total protein and catalase activity for all strains and compared total protein and catalase activity between starved and unstarved (control) cultures. WT E. coli undergoing amino acid starvation induced by excessive valine showed a 1.7-fold increase in catalase activity compared to the WT unstarved control. The non-overlapping 95% confidence intervals indicate that catalase was upregulated during amino acid starvation (Fig. 1). This result is consistent with previous studies that showed a similar increase in catalase activity when SR was successfully activated (17). Although catalase activity was higher in the relA strain under both starved and unstarved conditions compared to WT (Fig. 1), the change in catalase activity between starved and unstarved culture was less than that of WT (Fig. 2). The reduced ability of the relA mutant to produce catalase during starvation is due to its decreased ability to produce (p)ppGpp, as this mutant strain does not possess RelA. Similarly, the relA/spoT strain showed an even smaller difference in catalase activity compared to WT (Fig. 2), which is consistent with previous studies (10). This is a result of the relA/spoT mutant's inability to produce (p)ppGpp as this mutant strain lacks both RelA and SpoT (5). However, the reason behind the higher catalase in the two mutant strains compared to WT observed in Fig. 1 is not clear. This could be due to the differences in the genetic background of the mutant strains and the WT strain. The genotype of the relA strain and relA/spoT strain are Hfr(PO22), relA1, metB1 and Hfr(PO21), relA1, spoT1, metB1, respectively, whereas the genotype of the WT strain is F+, ΔrfbB51, F1-1, which is relatively different compared to the mutant strains (Supplementary Table 2). The mutant strains contain a mutation in metB1, a gene encoding a cystathionine gamma-synthase, which we believe may be the explanation behind the higher basal catalase activity observed in the two mutant strains compared to the WT. In a study performed in 2008, Hamelet et al. (18) showed that in mice deficient in cystathionine beta-synthase, liver catalase activity was upregulated. Although cystathionine gamma-synthase is not the same as cystathionine beta-synthase, their metabolic function is similar.

Results obtained from the Bradford assay were inconsistent with our expectation that the SR would cause a lower total cell protein concentration due to the decrease in synthesis of translational machinery (19). A higher protein concentration was found in the starved WT cells, whereas a lower protein concentration was found in the starved relA mutants (Fig. 3). No change in total protein concentration was found in the relA/spoT double mutant (Fig. 3). However, the high standard errors relating to the WT and relA/spoT mutant starved cultures make the results unreliable. Therefore, no conclusions on the effect of the SR on total cell protein concentration could be drawn and the results from the Bradford protein assay could not be used to characterize SR in this study. Even though the SR has been known to downregulate stable RNA-encoding genes and genes for growth, a large number of genes are also up-regulated including amino acid biosynthetic genes and genes for dealing with oxidative stress (20). The effect of the SR on gene expressions in the cell is complex; a large number of genes are involved in this stress response and most of these genes are still unknown. Hence, total protein quantification may not be a reliable indicator for measuring activation of SR.

MIC assay was used to evaluate long-term antibiotic resistance. Although there was at least a 1-fold difference in MIC across strains given the same antibiotic, no difference in MIC was observed between starved and unstarved cultures across strains. This indicates that the SR induced by amino acid starvation did not provide long-term tolerance to antibiotics compared to unstarved control cultures. Previous studies looking at adaptive kanamycin resistance upon the induction of SR also came to the same conclusion (13, 17).

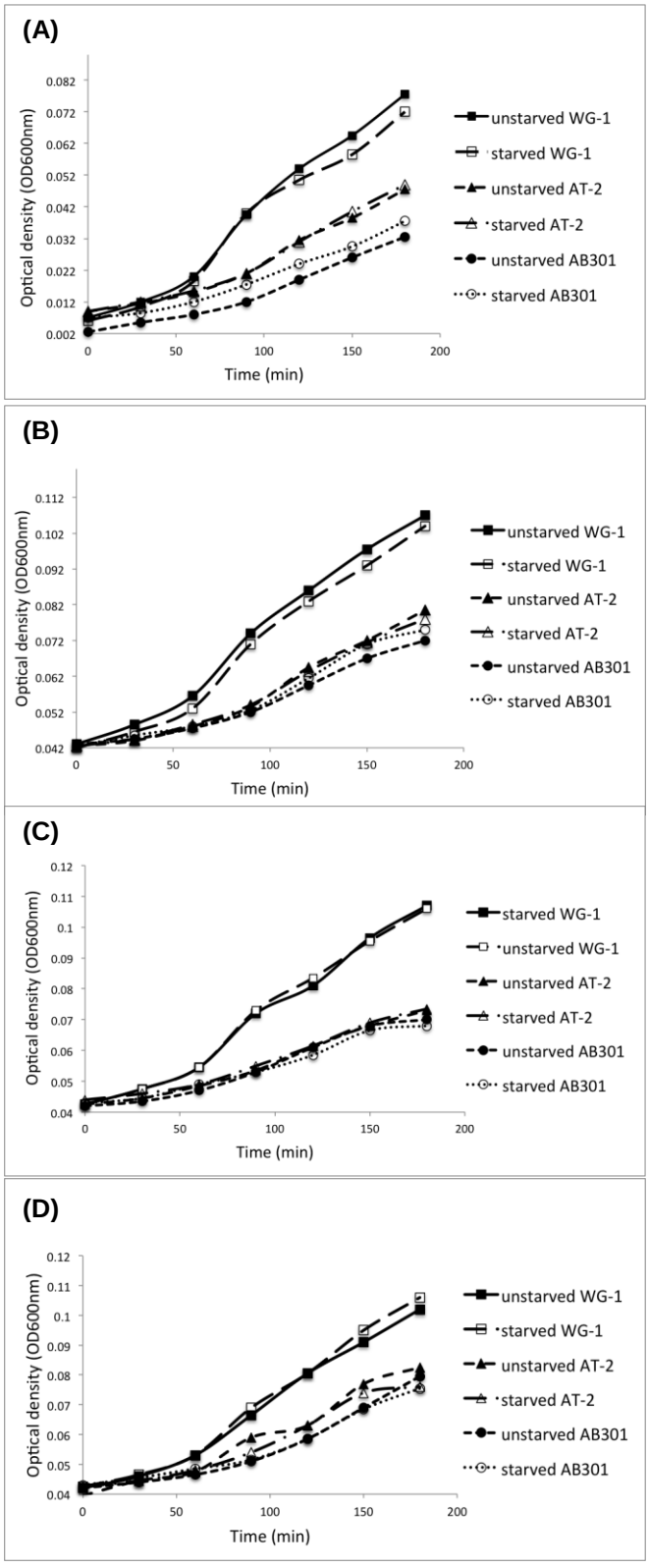

FIG 4. Growth curves of starved and unstarved cultures of *E.coli* WG1 (WT), AT-2 (*relA*), and AB301 (*relA/SpoT*) strains showing changes in turbidity of each culture over a 3 hour growth period (2 replicates per culture). **(A)** Cultures grown in the absence of antibiotic (controls). **(B)** Cultures grown in the presence of 1.25 µg/mL of Ampicillin. **(C)** Cultures grown in the presence of 2.5 µg/mL of Kanamycin. **(D)** Cultures grown in the presence of 3.91 ng/mL of Ciprofloxacin.

correlation between SR and increased antibiotic tolerance, growth curves were generated. The results showed that there was no difference in E. coli growth rate between the two nutrient conditions. It was believed that a reduction in growth rate would be observed when SR was activated in the WT and relA mutant strains during amino acid starvation since (p)ppGpp can be synthesized in these strains. A decrease in growth rate should be observed during the unfavourable starved condition. However, after 3 hours of monitoring, growth patterns of starved and unstarved cells were highly similar in our control condition in which there was no antibiotic added (Fig. 4). Traxler et al. (21), who also studied ppGpp-mediated SR in response to amino acid starvation, revealed a reasonable explanation for our unexpected results for the growth patterns of WT and relA mutant strains. They found that growth only begins to slow in WT culture at OD600nm of ~0.4 during a deprivation of amino acid (21). WT cultures were monitored for 3 hours and the OD600nm reading only reached 0.0775 at the final time point. According to their findings, the amount of (p)ppGpp might not be sufficient enough to result in a significant reduction in growth rate in the WT starved culture during the control condition. A similar explanation was proposed for our relA mutant strain when it was incubated in the absence of antibiotics. Since Durfee et al. (20) demonstrated a compromised ability of their relA mutant strain to induce SR during serine starvation, we reasoned that a higher OD600nm (OD600nm >0.4) was required for our starved relA mutant strain to display a remarkably slower growth rate. During amino acid starvation, our relA mutant cultures only reached an OD600nm of 0.049 in the control condition, suggesting that the amount of (p)ppGpp accumulated might not be sufficient to display a slower growth rate again.

Besides the unexpected results obtained in our control condition, unforeseen patterns were observed when cultures were incubated with a sub-MIC of kanamycin, ciprofloxacin and ampicillin. Higher tolerance to antibiotics in WT starved culture and relA mutant starved culture was expected compared to the corresponding unstarved cultures. In agreement with our expectations, Levin and Rozen (22) stated that the key mechanism resulting in antibiotic tolerance is the inactivity of antibiotic targets during the starvation-induced growth arrest. However, a higher tolerance to all three tested antibiotics in the WT and relA starved cultures was not observed. The OD600nm readings at the final time point, which were far lower than the required OD600nm suggested by the literature, could serve as a reason why starved cultures did not show higher antibiotic tolerance. There was not enough (p)ppGpp to lead to the inactivity of antibiotic targets; thus, there was no increased antibiotic tolerance.

A second explanation for the unexpected similar growth pattern displayed in WT and relA mutant cultures was suggested in a study conducted by Fung et al (23). They noted that bacterial tolerance to antibiotics is a summative result of their response to various nutrients (23). While Ji et al. (10) demonstrated that amino acid starvation could lead to increased antibiotic tolerance, the relative abundances of carbon source and nitrogen source ammonium salts, phosphate, and nucleobases could result in differential antibiotic tolerance phenotype. Different from the M9 media used by Ji et al., in which media was not supplemented with any amino acid sources, the M9 media in this experiment was supplemented with various amino acids and biotin to compensate for the known multiple auxotrophy of the relA/spoT mutant (21). This difference in media composition may have a negative effect on tolerance development.

Finally, we questioned the acceptability of using growth rate to characterize drug susceptibility. When observing a number of different cultures that shared identical growth rate, Fung et al. found a significant variation in drug tolerance phenotypes (23). Growth rate did not necessary correlate to survival rate in the presence of antibiotics in their study. Therefore, growth rate measured in this study might not reflect antibiotic tolerance.

In conclusion, activation of SR could be characterized by an increase in catalase activity in WT and relA mutant under starvation. However, no apparent relationship between SR and total cell protein concentration was found. Results from this study indicate that SR does not confer long-term antibiotic resistance to ciprofloxacin, ampicillin, and kanamycin. Nevertheless, whether or not there is short term tolerance in WT and mutant strains remains to be elucidated.

FUTURE DIRECTIONS

At early stages of the project, preliminary culture preparation involved 0.1 mg/mL L-Valine with 1.5 hours of incubation, as used in previous studies (10, 17). However, corresponding catalase results showed no difference in catalase production under starvation across strains. As a result, starvation conditioned were modified, increasing both the concentration in L-Valine and incubation time. However, whether or not this modified condition is the optimal condition for inducing stringency is unknown. To determine the optimal condition of inducing stringency, additional experiments testing variable concentrations of L-Valine and variable incubation times could be performed.

The SR is characterized by decreased transcription and increased catalase activity (10). However, due to the low accuracy and reproducibility of catalase quantification using this assay, a better method of quantifying catalase activity could be used. One option is a colorimetric assay, such as the amplex red stain assay that is commercially available as kits, which may provide more accurate and consistent results compared to the floating disc assay since it relies on measurements by spectrophotometry as opposed to experimenter observation.

As well, we have quantified total protein in an attempt to characterize SR in this project, but this method was not shown to be useful. Instead, RNA can be quantified as suggested by Brooks et al (24). As SR has been shown to directly affect synthesis of translational machinery, a decrease in RNA level is speculated to be mediated by increased levels of (p)ppGpp (24). RNA can be extracted and quantified using RT-qPCR.

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REFERENCES

1. **Potrykus K, Cashel M.** 2008. (p)ppGpp: still magical? Annu Rev
2. Microbiol **62**:35-51.
3. **Ferullo DJ, Lovette ST.** 2008. The stringent response and cell cycle arrest in *Escherichia coli*. PLoS Genet **4**(12): e1000300. doi: 10.1371/journal.pgen.1000300.
4. **Rodionov DG, Ishiguro EE.** 1995. Direct correlation between overproduction of guanosine 3', 5'-bipyrophosphate (ppGpp) and Penicillin Tolerance in *Escherichia coli*. J Bacteriol **177**(15):4224-4229.
5. **Boutte CC, Crosson S.** 2013. Bacterial lifestyle shapes stringent response activation. Trends Microbiol **21**:174-180.
6. **Hauryliuk V, Atkinson GC, Murakami KS, Tenson T, Gerdes K.** 2015. Recent functional insights into the role of (p)ppGpp in bacterial physiology. Nat Rev Microbiol **13**(5):298.
7. **Poole K.** 2012. Bacterial stress responses as determinants of antimicrobial resistance. J Antimicrob Chemother **67**(9):2069.
8. **Khakimova M, Ahlgren HG, Harrison JJ, English AM, Nguyen D.** 2013. The stringent response controls catalases in *Pseudomonas aeruginosa* and is required for hydrogen peroxide and antibiotic tolerance. J Bacteriol **195**(9):2011-2020.
9. **Dwyer DJ, Kohanski MA, Collins JJ.** 2009. Role of reactive oxygen species in antibiotic action and resistance. Curr Opin Microbiol **12**:482-489.
10. **Collins JJ, Dwyer DJ, Kohanski MA.** 2010. How antibiotics kill bacteria: from targets to networks. Nat Rev Microbiol **8**:423-435.
11. **Ji J, Kan S, Lee J, Lysakowski S.** 2013. Antibiotic tolerance in *Escherichia coli* under stringent response correlates to increased catalase activity. J Exp Microbiol Immunol **17**:40-45.
12. **Jung IL, Kim IG.** 2003. Transcription of *ahpC, katG, and katE* genes in *Escherichia coli* is regulated by polyamines: polyamine-deficient mutant sensitive H_2O_2-induced oxidative damage. Biochem Biophys Res Commun **301**:915-922.
13. **Cohen GN.** 2010. Biosynthesis of amino acids derived from phosphoglyceric acid and pyruvic acid, p 347-361. Microbial Biochemistry, 2nd ed. Springer Netherlands, Dordrecht, Netherlands.
14. **Chiang V, Wong B, Wong C, Yu J.** 2011. On the limited role of *relA* in kanamycin and amino acid starvation induced stringency and subsequent antibiotic protection in *Escherichia coli*. J Exp Microbiol Immunol **15**:59-63.
15. **Bansal S, Tandon V.** 2011. Contribution of mutations in DNA gyrase and topoisomerase IV genes to ciprofloxacin resistance in *Escherichia coli* clinical isolates. Int J Antimicrob Agents **37**:253-255.
16. **Hsieh S, Hwang S, Kim K, Kim S**. 2004. Investigation of the potential UV-A enhanced toxicity and the potential inhibition of catalase by phthalate exposed to UV-A. J Exp Microbiol Immunol **12**:50–56.
17. **Bio-Rad Laboratories**. Bio-Rad Protein Assay. http://www.bio-rad.com/LifeScience/pdf/Bulletin_9004.pdf.
18. 17. **Chen J, Chow B, Hopkins P, Tio J.** 2014. Adaptive kanamycin resistance induced by the stringent response in *Escherichia coli* is not dependent on *katE* and *katG*. J Exp Microbiol Immunol **18**:48-53.
19. **Hamelet, J, Seltzer, V, Petit, E, Noll, C, Andreau, K, Delabar, JM, Janel, N.** 2008. Cystathionine beta synthase deficiency induces catalase-mediated hydrogen peroxide detoxification in mice liver. Biochim Biophys Acta **1782:**482-488.
20. **Wendrich TM, Blaha G, Wilson DN, Marahiel MA, Nierhaus KH.** 2002. Dissection of the mechanism for stringent factor RelA. Mol Cell **10**:779-788.
21. **Durfee T, Hansen AM, Zhi H, Blattner FR, Jin DJ.** 2008. Transcription profiling of the stringent response in *Escherichia coli*. J Bacteriol **190**(3):1084-1096.
22. **Traxler MF, Summers SM, Nguyen H, Zacharia VM, Hightower GA, Smith JT, Conway T.** 2008. The global ppGpp-mediated stringent response to amino acid starvation in *Escherichia coli*. Mol Microbiol **68**(5):1128-1148.
23. **Levin BR, Rozen DE.** 2006. Non-inherited antibiotic resistance. Nat Rev Microbiol **4**(7):556-62.
24. **Fung DKC, Chan EWC, Chin ML, Chan RCY.** 2010. Delineation of a bacterial starvation stress response network which can mediate antibiotic tolerance development. Antimicrob Agents Chemother **54**(3):1082-1093.
25. **Brooks A, Yau J, Pham S.** 2011. Stringent response changes cell membrane permeability in *Escherichia coli* but does not develop cross tolerance to kanamycin, tetracycline and ampicillin. J Exp Microbiol Immunol **15**:30-35.