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Stringent response induced by amino acid starvation not involved in mediating broad spectrum antibiotic resistance in *Escherichia coli*

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SUMMARY Antibiotic resistance is a global threat to human welfare. The widespread use of antibiotics combined with a stagnation in the field of antibiotic development and discovery has exacerbated the problem of antibiotic resistance. It has therefore become essential to understand evolutionarily conserved cell response pathways and their contributions to antibiotic resistance to overcome this health crisis and develop new, more effective therapeutics. One such conserved pathway is the stringent response. The stringent response allows bacteria like *Escherichia coli* to survive adverse conditions by causing a diversion of resources away from growth and division and toward prolonged survival. Previous research has demonstrated that activation of the stringent response in *E. coli* results in increased resistance to certain antibiotics, such as kanamycin. In this study, we investigated the effect of the stringent response on resistance to a variety of antibiotics in *E. coli* using wild type and a Δ*relA*/Δ*spoT* mutant strains, as the Δ*relA*/Δ*spoT* mutant is unable to elicit the stringent response. Five classes of antibiotics with diverse cellular targets including peptidoglycan synthesis, gene expression, and outer membrane stability were selected. We hypothesized that WT *E. coli* undergoing stringent response activation will experience a broad spectrum increase in antibiotic resistance when compared to the Δ*relA*/Δ*spoT* mutant. Our results failed to replicate previous findings, refuted our hypothesis, and demonstrated that stringent response activation does not lead to a broad spectrum increase in antibiotic resistance.

INTRODUCTION

With the continual increase in the threat of antibiotic resistant pathogens, the American Center of Disease Control predicts approximately 35,000 American annual fatalities from associated conditions (1). As a result, it is becoming increasingly important to understand the mechanisms of antibiotic resistance in order to combat this problem. One such mechanism of interest is the stringent stress response. Due to the rising numbers of antibiotic resistant pathogens, the effect of the stringent response on resistance should be investigated in more detail. Environmental stresses such as nutrient starvation, heat shock, and antibiotic exposure can elicit the induction of the stringent response in bacterial cells, including *Escherichia coli* (2). The stringent response consists of widespread transcriptional and translational changes in bacterial cells, favoring prolonged survival over growth and reproduction (2,3).

Previous research has demonstrated that activation of the stringent response through nutrient starvation is correlated with an increase in resistance to the antibiotic kanamycin (4). Bactericidal antibiotics, such as kanamycin, have been associated with an increase in aerobic respiration which generates reactive oxygen species (ROS) within the cell (4). These reactive oxygen species are known to be harmful to cellular structures (4). As part of the stringent response in *E. coli*, oxidative control proteins, such as catalase, are upregulated to protect the cell from oxidizing agents, such as ROS. Upregulation of oxidative control proteins allows for the testing of stringent response activation by using a catalase test (5, 6).

Activation of the stringent response can result from exposure to a variety of stressors including heat shock and nutrient starvation (2). In *E. coli*, growth in the presence of excess L-valine inhibits isoleucine biosynthesis which activates the stringent response within the cell causing widespread transcriptional changes involving up to 500 genes (3, 7). Stress

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conditions are typical of antibiotic treatment in the gut, which may induce antibiotic resistance via the stringent response. A better understanding of this phenomenon could lead to more effective therapeutics and better antibiotic regimens.

It has been previously described that the accumulation of the global regulator guanosine tetraphosphate or ppGpp is directly linked to the activation and propagation of the stringent response under stress conditions (8). ppGpp is known to decrease the synthesis of stable RNA within cells causing a global transcriptional change and decrease in cellular metabolism (8). ppGpp is synthesized during nutrient starvation and is regulated by the gene products of *relA* and *spoT* (9). Both RelA and SpoT are involved in synthesis reactions, combining ATP and GTP to form ppGpp. A second regulatory mechanism of SpoT is the hydrolysis of ppGpp resulting in the production of GTP and pyrophosphate (10).

Previous research has acknowledged that amino acid starvation causes *relA* activation, resulting in downstream production of ppGpp (9). In addition, carbon source starvation is also known to increase *spoT* activity resulting in further synthesis and hydrolysis of ppGpp (9, 11). It has been previously demonstrated that most of these transcriptional changes resulting from altered ppGpp levels include the downregulation of highly-expressed rapid growth genes, such as those involved in cell wall biosynthesis, and the upregulation of genes involved in DNA replication inhibition (12).

The aim of this study was to investigate the effect of the stringent response on antibiotic resistance in *E. coli* by screening antibiotics from various classes. Resistance to these antibiotics was determined using a combination of both minimum inhibitory concentration (MIC) assays and IC50 values. We hypothesized that *E. coli* undergoing the stringent response would experience broad spectrum increase in antibiotic resistance. This increase in antibiotic resistance is linked to the broad nature of transcriptional and translational changes, including downregulation of rapid growth and developmental genes that are characteristic of the stringent response.

METHODS AND MATERIALS

Bacterial strains, growth conditions and confirmation. *E. coli* strains BW25113 (WT) and SL11W447-4 (Δ*relA::kan/*Δ*spoT::cam*) (Δ*relA/*Δ*spoT* double mutant*)* were obtained from the MICB 447/421 laboratory stocks of the Department of Microbiology and Immunology at The University of British Columbia. The strains were grown overnight in 5.0 mL Luria Bertani (LB) broth (1.0% w/v tryptone, 0.5% w/v yeast, and 1.0% w/v NaCl) at 37°C on a shaker at 200 rpm. Where applicable, a concentration of 0.1 mg/mL of L-valine was added to induce the stringent response. The Δ*relA/*Δ*spoT* mutant was verified by growth for 24 hours at 37°C on LB agar (1.0% w/v tryptone, 0.5% w/v yeast, 1.0% w/v NaCl, and 2.0% w/v agar) supplemented with 50 μg/mL kanamycin (KAN) and 25 μg/mL chloramphenicol (CAM) to assess the presence of antibiotic resistance cassettes. Further verification was done through PCR using primers that flanked *relA* and *spoT* genes from Cau et al. (13). The PCR products were run on a 2% agarose gel and Sanger sequenced to confirm the deletion of the *relA* and *spoT* genes.

Growth curves. Starved and unstarved (+/- 0.1 mg/mL L-valine) cultures of wild type (WT) and Δ*relA/*Δ*spoT* mutant were grown overnight in LB broth at 37°C and normalized to an OD_{600} of 0.015 corresponding to an exponential phase density. The cultures were plated in duplicate into a 96 well plate and growth measurements were made at 10 minute intervals for 16 hours to evaluate any growth defects in the Δ*relA/*Δ*spoT* mutant. The plate was incubated at 37°C and set at continuous orbital shake at a frequency of 282 CPM using a BioTek Epoch 2 Microplate Reader.

Catalase test. A catalase test was used to evaluate catalase activity and verify the activation of stringent response under starved and unstarved conditions. Wild type and Δ*relA/*Δ*spoT* mutant were grown overnight in LB broth or modified M9 minimal media (10% v/v M9 salts, 0.2% v/v 1 M $MgSO_4$, 0.01% 1 M $CaCl_2$ solution and 0.5% v/v glucose solution) at 37°C (+/- 0.1 mg/mL L-valine) prior to the assay (7). A loopful of each bacterial culture was transferred onto a clean, sterile glass slide and allowed to air dry. 2-3 drops of 3% (v/v) hydrogen peroxide (H_2O_2) were added onto the bacterial smear. A positive or negative

result was determined through observation of the presence or absence of a bubbling reaction, respectively.

MIC assay & IC50 analysis. A minimum inhibitory concentration (MIC) assay in LB broth was performed on the WT and Δ*relA*/Δ*spoT* mutant under starved and unstarved conditions (+/- 0.1 mg/mL L-valine). Starved and unstarved cultures of both strains were grown overnight in 5.0 mL LB broth at 37°C and shaking at 200 rpm. OD_{600} was measured and standardized to 0.015. Stock solutions of ampicillin (50 mg/mL, in water), tetracycline (20 mg/mL, in water), polymyxin B (30 mg/mL, in water), streptomycin (20 mg/mL, in water) and erythromycin (50 mg/mL, in ethanol) were prepared, sterilized using 0.22-µm filters, and stored at -20°C. Each antibiotic was diluted in 10 mL of LB broth (+/- L-valine) on the day of the assay. A final concentration of 120 µg/mL ampicillin, 200 µg/mL tetracycline, 20 µg/mL polymyxin B, 280 µg/mL streptomycin and 6000 µg/mL erythromycin were 1/2 serially diluted in their respective media (LB +/- L-valine). To each well of the 96-well plate, 180 µL of OD_{600} normalized culture and 20 µL antibiotic dilution were added. After incubation at 37°C for 16 hours, OD_{600} was measured. The MIC was reported by examining the lowest concentration of antibiotic that inhibited growth (**Table S1**). Growth inhibition was determined by the well that displayed a decrease in OD_{600} of at least two times than that of the previous well. In the cases where no minimum two times difference in OD_{600} was observed, MIC values were not reported. IC50 or the concentration of the antibiotic at which growth of the population is inhibited by 50% was determined by using KaleidaGraph software and obtaining sigmoidal best fit curves for each antibiotic and growth condition. The IC50 values for each sample were determined by averaging the values of at least 2 replicates. The change in resistance was deemed to be significant when the IC50 concentrations between experimental conditions differed by more than 2-fold.

RESULTS

Confirmation that Δ*relA*/Δ*spoT* mutant lacks both *relA* and *spoT* genes. Through antibiotic selection and PCR, verified using Sanger sequencing, we identified the presence of *relA* and *spoT* in WT *E. coli* but not in the Δ*relA*/Δ*spoT* mutant. The deletion of *relA* and *spoT* in the Δ*relA*/Δ*spoT* mutant is associated with the insertion of resistance cassettes for kanamycin (KAN) and chloramphenicol (CAM). WT *E. coli* were unable to grow on LB agar plates supplemented with KAN and CAM (**Figure S1**). The Δ*relA*/Δ*spoT* mutant was able to grow on LB agar plates supplemented with KAN and CAM. This confirmed the presence of KAN and CAM resistance cassettes in the mutant construct (**Figure S1**).

Table 1. Excess L-valine led to increased catalase activity in WT *E. coli* but not the Δ*relA*/Δ*spoT* mutant. WT + L-valine cultures produced positive results for the catalase test in both LB and M9 media. WT without L-valine, Δ*relA*/Δ*spoT* + L-valine, and Δ*relA*/Δ*spoT* without L-valine produced negative results for the catalase tests in both LB and M9 media.

Media	WT		Δ*relA*/Δ*spoT*	
	No L-valine	L-valine	No L-valine	L-valine
LB	-	+	-	-
	-	+	-	-
M9	-	+	-	-
	-	+	-	-

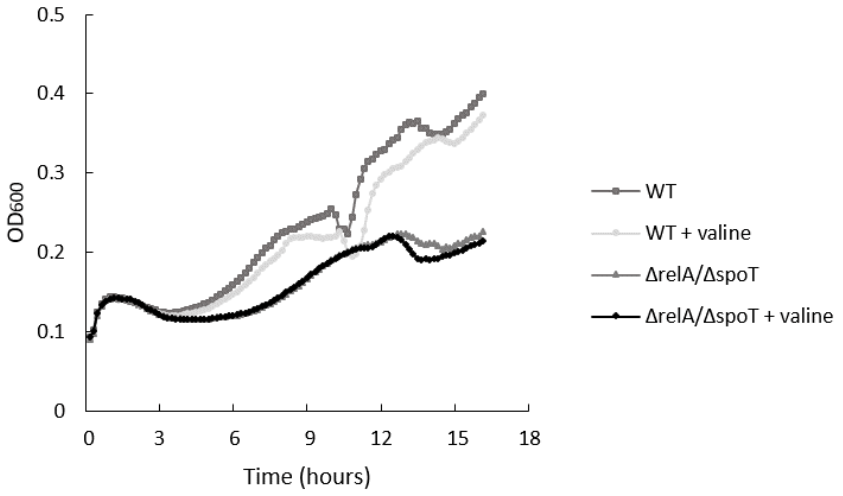

FIG. 1 The Δ*relA/*Δ*spoT* mutant exhibited decreased growth when compared to WT *E. coli*. OD_{600} measurements from 2 biological replicates (n=2) were averaged and plotted to generate the growth curves. The Δ*relA/*Δ*spoT* mutant exhibited decreased growth when compared to WT, regardless of the presence or absence of L-valine.

PCR amplification of *relA* and *spoT* genes confirmed the presence of *relA* and *spoT* in WT *E. coli* and the absence of *relA* and *spoT* in the Δ*relA/*Δ*spoT* mutant (**Figure S2A,B**). The amplified *relA* region in WT *E. coli* was approximately 600 base pairs (bp) in length. The amplified *spoT* region in WT *E. coli* was approximately 150 bp in length. When primers specific to *relA* were used, non-specific amplification was observed in the Δ*relA/*Δ*spoT* mutant (**Figure S2A**). Sanger sequencing confirmed the identity of the PCR amplicons from WT *E. coli* as *relA* and *spoT* (**Figure S2C,D**).

Excess L-valine induces the stringent response in WT *E. coli*. A catalase test was performed to determine if the stringent response was activated in WT and Δ*relA/*Δ*spoT* mutant. All Δ*relA/*Δ*spoT* mutants tested negative for the catalase test regardless of the presence or absence of L-valine in the growth media (**Table 1**). This was expected because *relA* and *spoT* gene deletions prevent the activation of the stringent response. WT grown in liquid LB or M9 media without the addition of L-valine produced negative results for the catalase test, indicating the stringent response was not activated (**Table 1**). All WT samples supplemented with L-valine produced positive results for the catalase test, indicating activation of the stringent response (**Table 1**). Based on these results, the addition of L-valine to the growth media was able to induce the stringent response in WT, but not in the Δ*relA/*Δ*spoT* mutant.

Growth deficiency observed in Δ*relA/*Δ*spoT* mutant. The WT and Δ*relA/*Δ*spoT* mutant were grown in LB media, with or without the addition of L-valine, to examine the effect of L-valine on growth. Both the WT and WT + L-valine samples exhibited similar growth curves, with WT+L-valine samples producing slightly lower OD_{600} values. (**Figure 1**). Δ*relA/*Δ*spoT* and Δ*relA/*Δ*spoT* + L-valine cultures showed similar growth over the 16-hour time period (**Figure 1**). An initial decrease in growth was observed in all samples, regardless of the *E. coli* construct or presence of L-valine (**Figure 1**). A decrease in growth for WT, but not Δ*relA/*Δ*spoT* samples was observed in the presence and absence of excess L-valine. This decrease in growth occurs approximately 10 hours into sample growth (**Figure 1**). The Δ*relA/*Δ*spoT* samples produced lower OD_{600} values than WT samples, indicating the Δ*relA/*Δ*spoT* mutant had decreased growth in LB media when compared to WT samples. (**Figure 1**).

Addition of excess L-valine led to decreased IC50 values for all antibiotics tested in both WT *E. coli* and the Δ*relA/*Δ*spoT* mutant. Minimum inhibitory concentration (MIC) assays and IC50 analyses were used to determine how the addition of excess L-valine and induction of the stringent response affects antibiotic resistance in WT *E. coli* and Δ*relA/*Δ*spoT* mutant. IC50 values were determined for ampicillin, streptomycin, polymyxin B, tetracycline, and erythromycin, both in the presence and absence of excess L-valine. The addition of L-valine to WT *E. coli* led to significant (>2 fold change) decreases in the IC50

values for polymyxin B and tetracycline; from 0.52 to 0.15 ng/mL (3.47 fold), and 7.52 to 3.1 µg/mL (2.43 fold) respectively (**Table 2**). Decreases in IC50 values for ampicillin, streptomycin, and erythromycin in WT *E. coli* after the addition of L-valine were not significant (**Table 2**). This trend was also apparent in the MIC values for WT *E. coli*, where the difference was less than 2 fold (**Table S1**). These trends suggest that the addition of excess L-valine, causing activation of the stringent response, decreased antibiotic resistance in WT *E. coli*.

The Δ*relA*/Δ*spoT* mutant, despite being unable to elicit a stringent response, also demonstrated a decrease in resistance to all antibiotics tested when L-valine was added. The addition of L-valine to the Δ*relA*/Δ*spoT* mutant led to a significant decrease in the IC50 values of ampicillin, streptomycin, and tetracycline by 3.63 fold, 2.66 fold, and 5.95 fold, respectively (**Table 2**). Non-significant decreases in the IC50 values for polymyxin B and erythromycin were observed (**Table 2**). In the Δ*relA*/Δ*spoT* mutant, the MIC value for ampicillin decreased from 3 µg/mL to 0.75 µg/mL with the addition of L-valine. The MIC value for streptomycin also decreased after the addition of L-valine, decreasing from 28 µg/mL to 3 µg/mL (**Table S1**). Overall, the Δ*relA*/Δ*spoT* mutant exhibited similar trends as WT *E. coli*, by exhibiting a decreased antibiotic resistance upon addition of L-valine.

DISCUSSION

Understanding the effect of the stringent response on antibiotic resistance is important due to the impacts it may have on therapeutics and drug regimens for treatment of pathogenic bacteria. The proposed idea of broad spectrum antibiotic resistance induced by the activation of stringent response comes from the diverse nature of changes which occur during the stringent response. These transcriptional and translational changes result in slowing down the replication and growth processes of bacteria. Induction of stringent response and high levels of ppGpp are linked to increased antibiotic resistance in a number of pathogenic bacteria including *Pseudomonas aeruginosa*, *Vibrio cholera*, *Staphylococcus aureus*, and *E. coli* (14). Optimizing the type and dose of antibiotic treatment will allow for consistent and effective clearance of pathogens, helping to slow the growing problem of antibiotic resistance.

In this study, we used minimum inhibitory concentration (MIC) assays and IC50 analyses to determine antibiotic resistance in *E. coli* under starved and unstarved conditions. The Δ*relA*/Δ*spoT* mutant exhibited significantly decreased growth when compared to WT

Table 2. Addition of L-valine led to decreased IC50 values in WT *E. coli* and Δ*relA*/Δ*spoT* mutants. The addition of L-valine significantly decreased (> 2-fold change) the IC50 values for polymyxin B and tetracycline in WT *E. coli*. Decreases in the IC50 values for ampicillin, streptomycin, and erythromycin were observed in WT *E. coli*, but these changes were non-significant. Significant decreases, associated with the addition of L-valine, were observed for ampicillin, streptomycin, and tetracycline in Δ*relA*/Δ*spoT* mutants. Non-significant changes in IC50 values were observed for polymyxin B and erythromycin. Significant results representing a greater than 2-fold change in IC50 values are represented in **bold**.

	WT		**Δ*relA*/Δ*spoT***	
	No L-valine	**L-valine**	**No L-valine**	**L-valine**
Ampicillin (µg/mL)	0.64	0.34	**0.69**	**0.19**
Streptomycin (µg/mL)	12.47	6.55	**4.98**	**1.87**
Polymyxin B (ng/mL)	**0.52**	**0.15**	0.75	0.54
Tetracycline (µg/mL)	**7.52**	**3.1**	**11.01**	**1.85**
Erythromycin (µg/mL)	190.9	146.55	96.3	65.32

under both starved and unstarved conditions which can be attributed to a previously characterized growth defect associated with the deletion of *relA* and *spoT* genes in *E. coli* (13). Induction of stringent response was observed in WT *E. coli* supplemented with excess L-valine. Additionally, increased catalase activity was also observed, consistent with previous research indicating that stringent response activation is associated with an upregulation in catalase production (15). However, induction of the stringent response in WT *E. coli* led to decreased IC50 values for all antibiotics tested, with 40% (2/5) of the changes being significant. These trends contradict previous research that has linked stringent response activation to increased antibiotic tolerance in *E. coli* (11). Similar trends were observed in the Δ*relA*/Δ*spoT* mutant suggesting that the decrease in IC50 values is linked to addition of excess L-valine. It is possible that the concentration of L-valine added was toxic to the cells. High concentrations of L-valine are known to induce a number of changes surrounding gene regulation and active transport in *E. coli* (16). L-valine addition can also lead to growth inhibition in *E. coli* (16). However, there was no clear evidence of L-valine toxicity based on the growth curve experiments performed. These results indicate that L-valine may have a different mechanism for changing the resistance of WT and Δ*relA*/Δ*spoT* mutants. De Felice et al. proposed that the severe excess of a compound, such as L-valine, does not accurately represent naturally occurring conditions and often results in changes beyond a cell's normal regulatory capacity (16). This is a possible reason for the observed decrease in IC50 values for WT and Δ*relA*/Δ*spoT* mutant *E. coli*. Excess valine is inducing the stringent response but is also influencing other aspects of the cell which are unable to be controlled by normal cellular regulatory mechanisms. These unexpected cellular changes are likely responsible for the decrease in IC50 values observed in WT and Δ*relA*/Δ*spoT* mutant *E. coli.*

Another explanation for the decrease in antibiotic resistance in WT *E. coli*, but not the Δ*relA*/Δ*spoT* mutant, is due the overproduction of ppGpp beyond normal cellular levels. As previously stated, ppGpp is an alarmone that is produced when the stringent response is activated (14). When normally produced in cells, ppGpp production and activation of the stringent response is linked to increased antibiotic resistance (14). However, the overproduction of ppGpp in cells is associated with decreased stable RNA synthesis (17). The lack of stable RNA synthesis, due to the severe excess of ppGpp, likely increased the susceptibility of *E. coli* to antibiotics as cells are experiencing significant cellular changes prior to antibiotic treatment.

Limitations Due to unforeseen circumstances, we were unable to complete this project as originally outlined and scheduled. For instance, MIC assay concentrations for some antibiotics could not be optimized. As a result, we did not have an equal number of biological replicates across all the antibiotics we chose to screen in this study. This prevented us from doing statistical analyses on antibiotics with a sample size of less than 3. However, we had enough data to perform an IC50 analysis on all the antibiotics. Moreover, in this study, we only tested one antibiotic per class. Using one antibiotic per class is not representative of the class due to modifications and variations present within the antibiotics in each class.

We also had to eliminate some antibiotics like ciprofloxacin, a fluoroquinolone, proposed in the original project, due to a lack of availability or inability of the antibiotic to dissolve in solvents like water or ethanol. Finally, we used 0.1 mg/mL L-valine to induce isoleucine starvation and stringent response in WT *E. coli*. This concentration of excess L-valine may have led to unintended cellular changes and therefore affected our ability to effectively draw conclusions about the role of the stringent response in antibiotic resistance.

One limitation with the Δ*relA*/Δ*spoT* mutant construct is that it contains antibiotic resistance cassettes for kanamycin and chloramphenicol. The presence of these cassettes could have influenced the Δ*relA*/Δ*spoT* mutant's resistance to other antibiotics, affecting our ability to draw conclusions regarding the role of *relA* and *spoT* induced stringent response on antibiotic resistance.

We used a catalase test to verify the activation of the stringent response in WT *E. coli*, analyzing the activation of the stringent response only at the phenotypic level and not directly through expression. Even though catalase activity is linked to stringent response

activity, there are better ways to measure the activation of the stringent response (4). For example, qPCR could have been used to determine the expression levels of *relA* and *spoT*. This would have allowed for us to quantify stringent response activation in each of the test conditions and connect the level of stringent response activation to the increase/decrease in antibiotic resistance.

Conclusions We demonstrated that the stringent response induced by excess L-valine in WT *E. coli* did not correspond to an increase in resistance to ampicillin, streptomycin, polymyxin B, tetracycline, or erythromycin. On the contrary, addition of L-valine to WT *E. coli* seemed to be linked to a significant increase in antibiotic susceptibility of the bacterial cells to tetracycline and polymyxin B. Moreover, addition of L-valine to the Δ*relA*/Δ*spoT* mutant also seemed to be associated with a significant increase in susceptibility to ampicillin, streptomycin and tetracycline. Our data contradicts past findings from other research projects where the stringent response was found to increase antibiotic resistance of WT *E. coli*. It is likely that this decrease in resistance was due to unintended cellular changes as a result of excess L-valine addition. Excess L-valine is known to cause changes in gene expression which may lead to an increase in cell's resistance to antibiotics.

Future Directions Activation of stringent response in *E. coli* has been previously shown to induce resistance to a broad range of antibiotics (4). However, due to time limitations, MIC assay concentrations could not be optimized for streptomycin, tetracycline and erythromycin. Future studies should aim to repeat MIC assays with these three antibiotics in addition to fluoroquinolones and aminoglycosides. Furthermore, due to a growth deficiency in the Δ*relA*/Δ*spoT* mutant, the MIC assays should be supplemented with an antibiotic disc diffusion assay to further verify the results.

In this project, we have considered only one antibiotic per class, for example, erythromycin from the class macrolides. Using one example from each class is not representative due to modifications and variations within the antibiotics. In addition, the results obtained from one antibiotic cannot be extrapolated to the entire class. Thus, it may be helpful to investigate the effect of stringent response on antibiotic resistance using 2-3 antibiotics from each class.

The catalase test was chosen as a method to verify the activation of stringent response in WT *E. coli* supplemented with L-valine based on time limitations and available resources. However, in the future, the stringent response should be verified by analyzing transcriptional changes in biosynthesis-related genes using RT-qPCR (2). Some possible candidates, in addition to *relA* and *spoT* genes, include *rpoH* and *bolA*, genes encoding a sigma factor and a protein involved in oxidative stress, respectively (2). It would be worthwhile to combine this verification approach with epifluorescence microscopy and DAPI staining to visualize the nucleoid morphology of cells undergoing the stringent response (18). During environmental stresses such as nutrient starvation, DNA is negatively supercoiled, decondensed, and incapable of replication (18, 19). Thus, when stained with DAPI, the nucleoid morphology of the cells undergoing the stringent response would appear decondensed and diffused, while cells growing in normal conditions would exhibit a nucleoid morphology that is more condensed and segregated (18).

In this study, we looked at the effect of stringent response on antibiotic resistance in *E. coli* by screening a broad range of antibiotics from different classes. However, the mechanisms of antibiotic resistance conferred by the stringent response still remain unclear. Therefore, antibiotic resistance and stringent response should be looked at a mechanistic level. Future studies should consider looking at the effect of stringent response on biosynthetic genes for a specific target of an antibiotic. One possible candidate includes polymyxin B and genes associated with the cell envelope.

Previous studies have demonstrated that overexpression of RelA protein results in an increase in cytoplasmic ppGpp concentration which inhibits stable RNA synthesis and is ultimately lethal to the bacterial cell (20). We used 0.1 mg/mL L-valine to induce isoleucine starvation and stringent response. However, it is likely that this concentration of L-valine over-induced the stringent response. Therefore, L-valine concentrations need to be adjusted and optimized to induce the stringent response more accurately in the future. Moreover, we

observed significant decreases in IC50 values in both WT and the Δ*relA*/Δ*spoT* mutant under starved conditions. Thus, future studies should also investigate the effect of L-valine on antibiotic resistance outside the effects of stringent response. It is likely that the addition of L-valine itself has an impact on antibiotic resistance.

To resolve the issue regarding the presence of kanamycin and chloramphenicol resistance cassettes in the antibiotic, these resistance cassettes should be excised from the genome of the Δ*relA*/Δ*spoT* mutant. This would prevent any unexpected complications from the presence of the resistance cassettes, allowing for more clear conclusions.

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