Journal of Experimental Microbiology and Immunology (JEMI) Vol. 22:1-9
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# Differences in growth and antibiotic susceptibility in *Escherichia coli* stringent response mutants are conferred in minimal nutritional conditions irrespective of amino acid limitation.

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**SUMMARY** The rise of antibiotic resistance crisis is a critical challenge facing healthcare providers around the world. To combat this phenomenon, we must understand underlying cellular processes that contribute to antibiotic resistance, such as the stringent response. The stringent response is a bacterial stress triggered signal transduction pathway that leads to global reprogramming at transcriptional and translational levels. The primary mediator of the stringent response is guanosine tetraphosphate (ppGpp), an alarmone that indirectly regulates essential processes for survival including amino acid biosynthesis, translational assembly and stress-associated proteins in response to environmental stimuli. In *Escherichia coli*, ppGpp levels are controlled by two proteins, RelA and SpoT. In this study, we characterized the growth and antibiotic susceptibility of single and double mutants of RelA and SpoT, to provide a future basis for characterization of the stringent response-controlled genes that mediate antibiotic resistance. A *relA/spoT* double mutant strain exhibited reduced growth compared to the wild type strain in M63B1 minimal medium with defined amino acid concentrations but not in Mueller-Hinton broth supplemented with excess valine. All strains displayed reduced overall growth in minimal medium. Minimum inhibitory concentrations of antibiotics were determined in Mueller-Hinton broth and M63B1 minimal medium with defined amino acid concentrations. Under the conditions tested, the stringent response mutants showed up to a 2-fold increase in antibiotic susceptibility. Further work is required to develop a robust strategy for activating the stringent response in future studies.

## INTRODUCTION

Antibiotic resistance is a clinical term to describe bacteria that acquire tolerance to normal doses of antibiotics that had previously been bactericidal or bacteriostatic. An alarming global phenomenon, rising rates of antibiotic resistance are jeopardising the clinical treatment of bacterial pathogens, especially in cases where bacterial pathogens have acquired resistance to multiple frontline antibiotics. The rapid proliferation of antibiotic resistance, attributed in part to overuse, is further complicated by a slowing in the discovery and commercialization of novel antibiotic classes (1).

Antibiotic resistance is grouped into three subcategories: intrinsic, acquired and adaptive. Intrinsic resistance is defined as the innate ability of a species to be unresponsive or resistant to antimicrobial agents (2). Acquired resistance arises when normally susceptible organisms incorporate new genetic material such as plasmids, transposons, naked DNA and integrons which encode for gene products that mediate resistance, or through genetic mutations conferring resistance. The third resistance mechanism is adaptive resistance, in which global changes to gene expression allow a bacterium to increase its resistance to antibiotics (3). While the mechanisms underlying adaptive antibiotic resistance are not fully understood, the stringent response (SR) has been implicated in helping to drive adaptation (4, 5). The SR is a stress induced signaling pathway characterized by a global reprogramming of cellular processes. In gram-negative bacteria, the SR has been shown to mediate adaptive resistance to multiple classes of antibiotics (10, 11, 12). SR mediated resistance occurs via changes to transcription profiles that lead to: target modification via a mutation or by specialized enzymatic changes, target substitution where the primary target of an antibiotic is replaced, antibiotic modification or destruction, and antibiotic efflux and restricted antibiotic permeability (6,18). Extensive work has therefore established an association between the SR

**Published Online:** 24 August 2018

**Citation:** Cau M, Goodall A, Liu L, Liu N. 2018. Differences in growth and antibiotic susceptibility in *Escherichia coli* stringent response mutants are conferred in minimal nutritional conditions irrespective of amino acid limitation. JEMI 22:1-9

**Editor:** Julia Huggins, University of British Columbia

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and adaptive antibiotic resistance but questions remain, such as which antibiotics lead to strong activation of the SR.

In gram-negative bacteria, the SR is mediated by the alarmone guanosine pentaphosphate (pppGpp), whose levels are controlled by two enzymes RelA and SpoT. RelA is a synthetase, synthesizing ppGpp and pppGpp from ATP through the transfer of a pyrophosphoryl group to GDP or GTP respectively. RelA is activated during amino acid starvation, when an uncharged tRNA enters the A site in the ribosome (6). SpoT is a bifunctional protein with both synthetase and hydrolase activity. Its production of ppGpp depends on signals relayed when iron, nitrogen, carbon, phosphate or fatty acids are scarce. The hydrolysis of ppGpp to GTP and PPi prevents accumulation of the alarmone. During nutrient scarcity, ppGpp causes global transcriptomic shifts by changing the binding affinity of RNA polymerase associated sigma factors for promoters (7, 8, 9).

Herein, we aimed to create a foundation on which to characterize the interaction between the SR and antibiotic resistance. Genomic deletions of *relA* or *relA* and *spoT* were confirmed, and the effect of these deletions on growth and antibiotic susceptibility was investigated. The results are discussed in terms of developing a suitable platform for the analysis of phenotypic characteristics in SR mutants.

## METHODS AND MATERIALS

**Bacterial strains, media, and growth conditions.** Bacterial strains used in this study are listed in Table 1. *E. coli* strains used in the study were cultured at 37 °C with shaking at 250 rpm in lysogeny broth (LB) f, double yeast tryptone (dYT), Mueller-Hinton broth (MHB), or M63B1 minimal media (100 mM $KH_2PO_4$, 0.8 mM $MgSO_4$, 3 µM Thiamine B1, pH 7.4 (15)) for 16-18 hours. M63B1 medium was supplemented with 0.4 % dextrose and 400 µM of all 20 proteinogenic L-amino acids for unstarved conditions or 60 µM of L-leucine and 400 µM of 19 other proteinogenic L-amino acids (13) to be used as starved conditions.

**Strain genotyping.** Primers for genotyping *relA* and *spoT* were designed using PrimerBLAST and listed in Table 2. Genomic DNA was isolated using the PureLink genomic DNA extraction kit (Invitrogen) as per manufacturer protocol. PCR reactions was performed in 50 µL volumes using Platinum Taq Polymerase (ThermoFisher) as per the manufacturer protocol. 20 ng of DNA was used as template. A thermocycler from BioRad was used, with an initial heating step of 94 $^0$C for 2 minutes, prior to a 94 $^0$C denaturation step, 53 $^0$C annealing step and 72 $^0$C elongation step for 30 cycles. A setting of 5 minutes at 72 $^0$C was implemented at the end to ensure thorough amplification and the products were held at 4 $^0$C until agarose gel electrophoresis. PCR products were visualized using a 2% w/v UltraPure$^{TM}$ Agarose gel (ThermoFisher) with 1x TAE and visualized using SYBR Safe (ThermoFisher).

**Growth Curve Excess Valine** Overnight *E. coli* cultures were harvested by centrifugation at 5.0 g for 3 minutes, and washed with MHB or M63B1 media to remove excess amino acid from overnight culture medium. Resulting cells were diluted in MHB or M63B1 with varying concentration of L-valine (Sigma) to a final optical density at 600 nm ($OD_{600}$) of 0.01 and 200 µL was transferred to a flat bottom 96-well polystyrene microtiter plate (Falcon). Resulting cultures were incubated at 37°C while shaking at 567 rpm in a microplate reader (Synergy H1; BioTek). $OD_{600}$ was measured over a 15-hour period. Experiments were performed with three biological replicates.

### Table 1 Bacterial strains used in the study

| Strain | Relevant characteristics or genotype[a] | Reference or source |
|---|---|---|
| ***Escherichia coli*** | | |
| BW25113 | Laboratory wild type strain | [23] |
| JW2755-3 | Keio Collection: *relA* deletion mutant, Kan resistance | [24] |
| SL11W447-4 | *relA*/*spoT* double deletion mutant, Kan/Cam resistance | [17] |
| PAO1.Δ*relA*/Δ*spoT* (complement *relA*) | *relA* deletion mutant, Kan resistance gene excised | [21] |
| PAO1.Δ*relA*/Δ*spoT* (complement *spoT*) | *relA*/*spoT* deletion mutant Kan/Cam resistance gene excised | [21] |

**Table 2 Primers used in the study**

| Primer | Sequence (5' – 3') |
|---|---|
| *relA_f* | CGATTACGTCGCTAACCCGA |
| *relA_r* | TGTCACGGTCCTGTTTACGG |
| *spoT_f* | CATTAAAGGTGCCGATGGCG |
| *spoT_r* | AGCCACGGATATTACGGCAG |

**Preparation of Antibiotics** Antibiotic stocks were created using colistin, erythromycin, gentamicin, kanamycin, amoxicillin, carbanecillin, aztreonam, amikacin, cefotaxime, tetracycline, and amoxicillin dissolved in appropriate solvents (16) at 10 mg/mL and filter sterilized using a 0.22 µm syringe filter (Fischer). Serial dilutions were performed to create 1 mg/mL, 0.1 mg/mL, and 0.01 mg/mL working solutions.

**Drug Susceptibility Assay** The minimum inhibitory concentrations (MICs) of antibiotics for *E. coli* wildtype and stringent response mutants was determined by the broth dilution assay in 96-well plates (22). Assays were performed in MHB broth, and in M63B1 unstarved and starved conditions. Bacterial growth of the overnight growth was examined by a visual inspection followed by a photometric measurement. All experiments were performed in at least triplicate, following the Clinical and Laboratory Standards Institute recommendations.

## RESULTS

**Genotype of *relA* single mutant and *relA*/*spoT* double mutants confirmed by PCR.** The confirmation of the appropriate genotypes was to prevent that incorrect strain identity would lead to mistakes in data interpretation of growth behaviour and antibiotic susceptibility. Five *E. coli* strains were used in this part of the study: JW2755-3, SL11W447-4, JKL112W-1, JKLL12W-2 and BW25113. *E. coli* JW2755-3 strain is commercially obtained strain from KEIO that is unmodified, containing an antibiotic resistance cassette in place of the entire *relA* gene; thus it is a *relA* single mutant. *E. coli* SL11W447-4 is a JEMI generated strain (17) that contains whole deletions in both *relA* and *spoT*, which are replaced by antibiotic resistance cassettes. It was generated by the excision of the *spoT* gene from JW2755-3 using the lambda red recombinase system. Both JW2755-3 and SL11W447 served as ulterior positive controls for genotype confirmation. *E. coli* JKL112W-1 is a *relA* single mutant produced by the excision of the Kanamycin antibiotic resistance cassette from *E. coli* JW2755-3 and *E. coli* JKL112W-2 is a *relA/spoT* double mutant where the antibiotic resistance cassettes for kanamycin and chloramphenicol were removed from the SL11W447-1 strain (21). Both are JEMI generated and obtained from JEMI stores. The wild type designated *E. coli* strain used was BW25113. As shown in Fig. 1a, only one defined band of 534bp was found at the location of wild type *E. coli*, indicating the clear excision of *relA* (size=534 bp) gene in all other bacterial strains. Similarly, *spoT* (size = 137bp) was found in all strains beside the double mutants (Fig. 1b). PCR amplification confirmed the correct deletions in the JKLL12W-1, JKLL12W-2, JW2755-3 and SL11W447-4 strains. The JKLL12W-1 and JKLL12W-2 strains, hereafter referred to as the *relA* single mutant and the *relA/spoT* double mutant respectively, were chosen for downstream experiments because they did not contain antibiotic resistance cassettes.

**Excess valine does not induce stringent response-associated growth arrest in rich or poor medium.** In order to observe differences in the phenotypes of stringent response mutants when compared to the wild type strain, the stringent response needs to be activated with a reliable method. Supplementation with excess valine treatment is a well described method of SR induction (19). This method exploits the common biosynthetic enzyme of isoleucine and valine, where the buildup of valine triggers a negative feedback loop that inhibits isoleucine synthesis and growth arrest is induced. However, the effect of excess valine on growth ranging from 0.5 mg/mL (Data not shown) to 4.0 mg/mL (Fig. 2), which has been previously employed to activate the SR pathway (14), did not induce growth arrest or a stationary phase-like growth profile in nutrient rich MHB. The growth profiles of

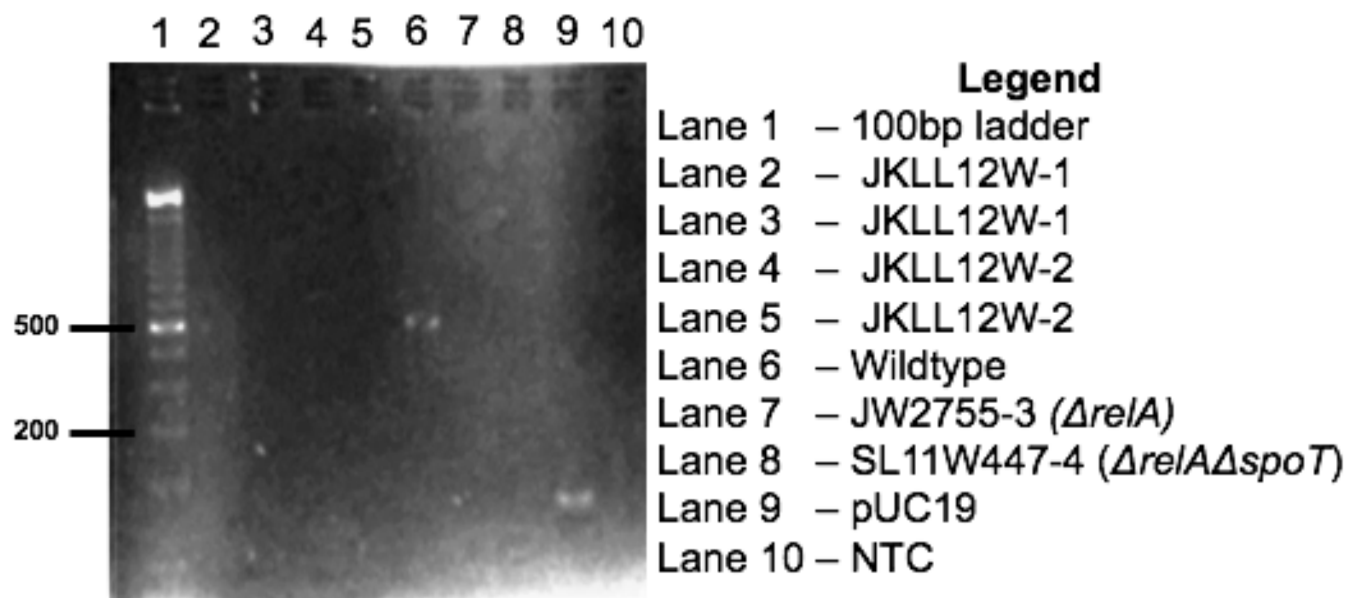


**FIG. 1A Deletion of *relA* is confirmed in all bacterial strains, excluding wild type *E.coli*.** pUC19 is used as positive control.

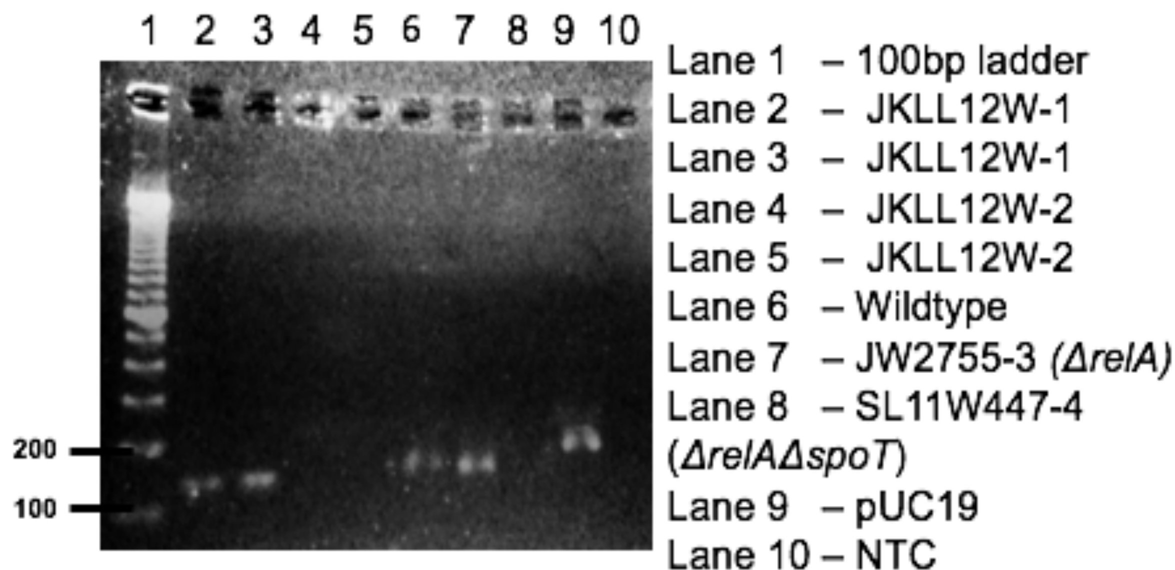


**FIG. 1B Deletion of *spoT* is confirmed in SL11W447-4 and JKLL12W-2 double knock-out strain**. pUC19 is used as positive control.

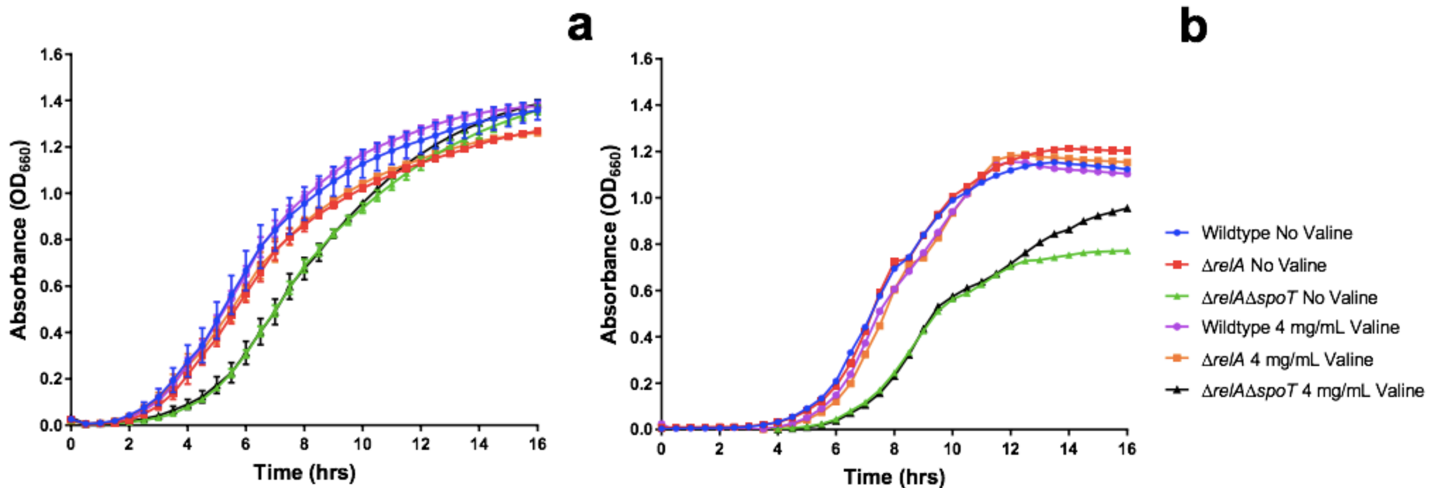


**FIG. 2 Growth profiles of BW25113, JKLL12W-1, and JKLL12W-2 in MHB (a) or M63B1 with excess valine (b). No change in the growth profile was shown amongst different strains.**

wildtype and mutants are comparable between high valine and no valine (Fig. 2). This is also true when excess valine is supplied to M63B1 minimal media (Fig. 2). These data suggest that valine supplementation was not sufficient to induce the SR in our experimental conditions. Larger studies of the stringent response commonly employ serine hydroxamate (SHX), an amino acid analog which binds and inhibits the aminoacylation of seryl-tRNA. This leads to accumulation of uncharged tRNA and induction of the stringent response. SHX was deemed an infeasible approach in our study due to its high cost but since differences in SR mutants with respect to the wildtype (WT) *E. coli* can only be documented under stringent conditions, definitive induction of the pathway precludes the study of downstream effects. Future JEMI contributors may opt for SHX as their method of choice.

**The growth profiles of SR single mutants are comparable to that of wild type in nutrient rich and poor media but *relA/spoT* double mutants exhibit a strong growth deficiency irrespective of amino acid contents**. It was thought that a synthetic medium where amino acids were supplemented to create the desired conditions represented an alternative approach to SR activation. In such an approach, the difference between a starved and an unstarved medium for stringent response induction was the concentration of isoleucine. Because the use of excess valine to indirectly promote isoleucine starvation was unsuccessful, here we opted for a direct strategy to induce the SR. In unstarved conditions, the growth curve of the *relA* single mutant was identical to wild type *E. coli.* Compared to wild type, the *relA*/*spoT* double mutants displayed a lower growth rate and yield, reaching a maximum $OD_{600}$ that was approximately 60% of the wild type strain and a lowered exponential phase slope with respect to the wild type (Fig. 3). The experiment was repeated with a lower concentration of isoleucine, and growth curves were again analyzed. All strains were negatively affected by the isoleucine deficiency. In a similar fashion to the amino acid rich M63B1 medium, the *relA* single mutant and the wild type strain had nearly identical growth curves albeit a maximum plateau of 36% relative to unstarved conditions in which isoleucine was not restricted. The *relA/spoT* double mutant was most severely impaired in growth as it reached 60% of the growth observed by the wild type bacteria in isoleucine poor M63B1 medium (Fig. 3). The phenotype displayed by the *relA/spoT* double mutant in amino acid rich conditions was unexpected and a growth deficit of the same magnitude was seen under amino acid starvation conditions. In summary, the use of M63B1 medium either supplemented with all amino acids at equal concentrations or with a decreased isoleucine concentration stunts bacterial growth and are suitable media for growth comparison of stringent response mutants.

**Table 3. MIC of wildtype, stringent response single mutant, and double mutants in MHB and M63B1 (unstarved and starved).** Values shown are in µg $mL^{-1}$.

| | MHB | | |
|---|---|---|---|
| | Wildtype | Δ*relA* | Δ*relA*Δ*spoT* |
| Amikacin | 0.16 | 0.16 | 0.16 |
| Amoxicillin | 1.25 | 0.63 | 0.63 |
| Aztreonam | 0.031 | 0.031 | 0.031 |
| Carbanecillin | 12.5 | 12.5 | 12.5 |
| Cefotaxime | 0.016 | 0.016 | 0.016 |
| Ciprofloxacin | 0.063 | 0.031 | 0.063 |
| Colistin | 0.16 | 0.16 | 0.16 |
| Erythromycin | 250 | 250 | 250 |
| Gentamycin | 1.25 | 1.25 | 1.25 |
| Kanamycin | 3.13 | 3.13 | 3.13 |
| Tetracycline | 2.50 | 2.50 | 2.50 |

| | M63B1 Unstarved | | | M63B1 Starved | | |
|---|---|---|---|---|---|---|
| | Wildtype | Δ*relA* | Δ*relA*Δ*spoT* | Wildtype | Δ*relA* | Δ*relA*Δ*spoT* |
| Amikacin | 0.63 | 0.63 | 0.63 | 0.63 | 0.63 | 0.63 |
| Amoxicillin | 1.25 | 0.63 | 0.63 | 1.25 | 0.63 | 0.63 |
| Aztreonam | 0.008 | 0.008 | 0.008 | 0.008 | 0.008 | 0.008 |
| Carbanecillin | 0.031 | 0.031 | 0.031 | 0.031 | 0.031 | 0.031 |
| Cefotaxime | >0.004 | >0.004 | >0.004 | >0.004 | >0.004 | >0.004 |
| Ciprofloxacin | 0.031 | 0.031 | 0.016 | 0.031 | 0.031 | 0.016 |
| Colistin | 0.31 | 0.16 | 0.16 | 0.31 | 0.31 | 0.16 |
| Erythromycin | 15.63 | 15.63 | 15.63 | 15.63 | 15.63 | 15.63 |
| Gentamycin | 1.25 | 1.25 | 1.25 | 1.25 | 0.63 | 0.63 |
| Kanamycin | 0.63 | 0.31 | 0.31 | 0.63 | 0.31 | 0.31 |
| Tetracycline | 5 | 2.5 | 2.5 | 2.5 | 2.5 | 1.25 |

**Minimum inhibitory concentration is lower in stringent response mutants for multiple classes of antibiotics when cultured in minimal media.** Previous work has established that the stringent response is strongly implicated in antibiotic resistance. Our goal was to compare SR single and double mutant strains to wild type bacteria under starved conditions to identify the antibiotics that the SR most strongly mediates resistance to. For multiple antibiotic classes, this information is unknown (10). Minimum inhibitory concentration assays using MHB medium showed differential susceptibility to amoxicillin between wildtype and SR mutants (Table 3). In minimal medium M63B1, one of the two or both stringent response mutants were 2-fold more susceptible to multiple classes of antibiotics which were amoxicillin, colistin, ciprofloxacin, kanamycin, and tetracycline (Table 3). Interestingly, while most of the MIC range of the antibiotics tested are identical between MHB and M63B1, erythromycin and kanamycin had a substantial decrease in of MIC in M63B1 compared to that of MHB. Furthermore, in wild type *E. coli* tetracycline was associated with a twofold reduction in MIC under the starvation condition in M63B1. We speculate that if the reduction was seen in the unstarved condition relative to the starved condition, the increased susceptibility could be SR driven.

## DISCUSSION

This study presents evidence confirming the genotype of previously constructed SR mutants and establishes methods for examining SR mutants under different conditions. More specifically, it determines the growth behaviour and antibiotic susceptibility of SR mutants in various growth media.

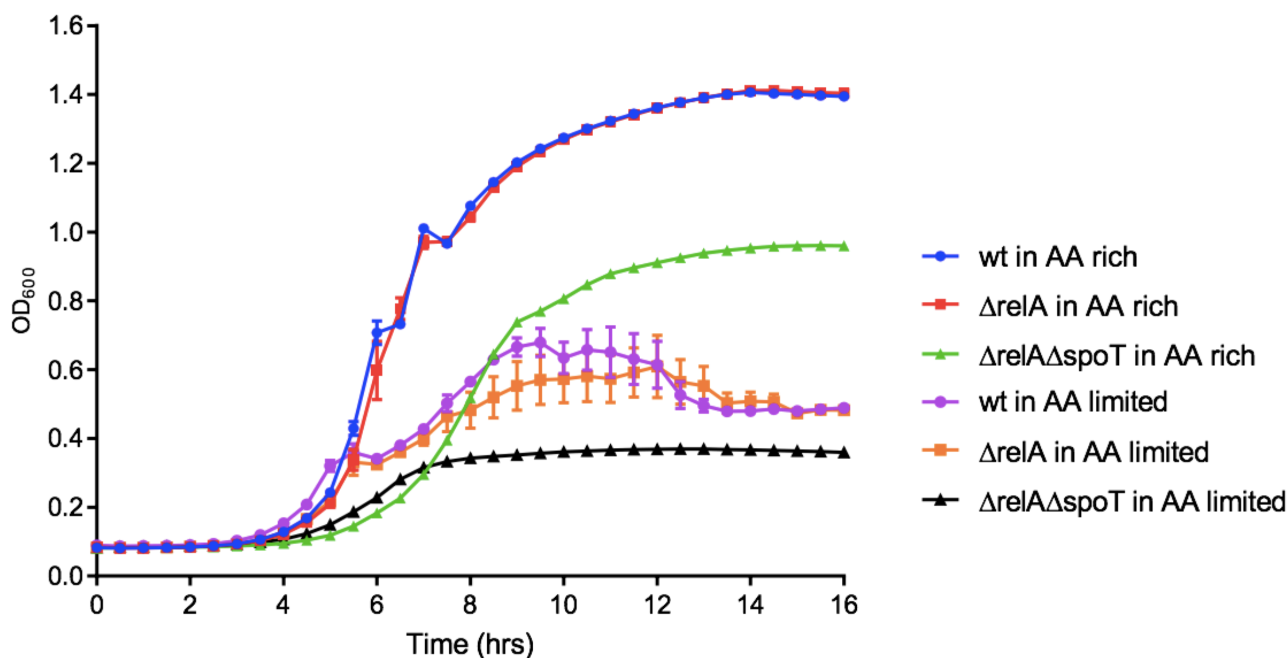


**FIG. 3** Growth profiles of BW25113, JKLL12W-1, and JKLL12W-2 in M63B1 supplemented with all amino acid at 400 $\mu$M or L-isoleucine limited to 60 $\mu$M. An early growth arrest observed in all strains under L-isoleucine limited condition using M63B1 medium. Intrinsic growth deficiency is also observed in the SR double mutant.

Recently, Oh et al. conducted a similar study using LB medium and 1% glucose in *Vibrio cholerae relA/spoT* mutants. In *V. cholerae*, the SR is controlled by 3 proteins. These are RelA, RelV and SpoT. RelA and SpoT perform analogous functions to their *E. coli* counterparts, and RelV also synthesizes ppGpp. It was found that *relA/spoT* double mutants grow to the same extent as wild type *V. cholerae* (20). In addition, a relA/relV/spoT triple mutant was defined as a $ppGpp^0$ mutant, and is SR deficient. The phenotype exhibited by this strain was a severe growth defect in LB media supplemented with 1% glucose. In our study, the M63B1 medium was supplemented with 0.4% glucose. Oh et al. reasoned that the accumulation of organic acids occurs as a result of incomplete glucose metabolism, which decreases the pH of the environment. *V. cholerae* thrives in media with a pH greater than 5.5. This explanation proved to be accurate as in wildtype and *relA/spot* double mutants the pH of the LB media initially dropped to 5 but then recovered to 8 whereas in a $ppGpp^0$ mutant this recovery was not seen. We did not measure the pH of our media but it is plausible that the adjustment of the pH in our study would have corrected for the growth deficiency observed. Unlike the double mutants, the *relA* single mutants remained at least as viable as wild type bacteria. Durfee et al. performed a similar experiment using rich media where *relA* mutants did not stop growing upon stringent response induction by SHX as opposed to wild type MG155 (18). This is consistent with expected phenotype of a *relA* mutant because consequently ppGpp is not synthesized, the SR is delayed and growth arrest does not promptly occur. A comparison of that study to the one here is not possible for two reasons; the authors used SHX, which is a failsafe method for SR induction, and also added their SR inducing compound when the optical density at 600 nm reached 0.2, which we did not do. We used excess valine or a direct isoleucine limitation for stringent response induction right from the beginning of the growth period. We also argue that the use of valine to induce the stringent response should be avoided, because growth arrest, the fundamental result of stringent response activation, did not occur. The experiment was performed in rich media and minimal media but all three strains displayed identical growth curves. We reasoned that since the mode of action of excess valine is isoleucine depletion, a suitable alternative would be to reduce isoleucine concentrations. It should be pointed out that the negative effect of the isoleucine restriction on wild type *E. coli* demonstrates that the devised method is not without merit.

The results taken together do not lead us to believe that the M63B1 medium along with isoleucine starvation created a true phenotypic difference between the mutants and the WT. This is because the deficiency was already present in nutrient rich conditions, suggesting that mutations in both SR genes are indeed detrimental but not dependent on stringent conditions. This is an unexpected result, because under non-stringent conditions the loss of the pathway seems irrelevant. That is, the environmental stimulus represented by isoleucine starvation in our study had no effect on a bacterial strain that lacked the genes to respond accordingly.

This study was limited to the identification of potential antibiotics to which the stringent response conferred resistance. In MHB, *relA/spoT* double mutants exhibited a two-fold increase in susceptibility to amoxicillin. Howeve,r as a whole, the data from the minimum inhibitory concentration assays were consistent with the results of the valine growth curves in that few trends were observed between SR mutants and wild type *E. coli* in rich MHB. When MHB was supplemented with excess valine for SR activation, the growth of all strains was similar. There was also no observed difference between the strains when MIC using M63B1 unstarved and starved media was compared. The SR was expected to be activated by nutrient scarcity in wild type strains and to display a greater magnitude of resistance with respect to pathway deficient mutants, but this was not found. However, the switch from MHB to M63B1 minimal media irrespective of isoleucine restriction produced several susceptibility differences between the genotypes. Stringent response mutants are up to two-fold more susceptible to amoxicillin, ciprofloxacin, colistin, kanamycin and tetracycline. Our findings corroborate those of previous authors, whose work highlighted the importance of the stringent response in resistance to the above-mentioned antibiotics (10, 11, 12).

**Conclusions** We characterized *relA* single mutants and *relA/spoT* double mutants through growth assays and antibiotics susceptibility assays and report no growth difference between wild type and mutants when grown in MHB media with the addition of excess valine, which is a well-known method to induce the SR. We manually supplemented a minimal medium M63B1 with amino acids to create starved and unstarved conditions, and determined that growth differences between the mutant strain and the wild type was not dependent on amino acid availability. However, we failed to provide evidence of differential growth between stringent response mutants and wild type. Stringent response mutants are up to two times more susceptible to Amoxicillin, Ciprofloxacin, Colistin, Kanamycin, and Tetracycline when grown in M63B1 minimal medium, but susceptibilities to these antibiotics was similar between starved and unstarved conditions. We conclude that the results seen using M63B1 minimal medium supplemented with amino acids for studying growth profiles of SR mutants are encouraging but need to be paired with more effective strategies to confirm SR activation. Furthermore, MIC data from stringent response mutants is in line with other studies suggesting that the SR mediates resistance to certain classes of antibiotics. Future work with those drugs is necessary to determine the mechanism by which this occurs.

**Limitations** This study has noteworthy limitations. Firstly, we were unable to directly measure stringent response activation. Because of this, we subjected the stringent response mutants to excess valine concentrations or reduced isoleucine concentrations in minimal medium and studied the growth profiles and antibiotic susceptibilities of these strains, where growth and susceptibility were used as indirect indicators of SR activation. The growth curves observed across strains in all conditions tested were concluded to be identical but we speculate that stringent response activation did not occur. However, because adaptive antibiotic resistance is known to be SR-dependent, the null effect of the SR inducing conditions used in our study could signify that the MIC difference is not due to the loss of the SR. This idea is supported by the observation that the wild type did not display any MIC difference under starved and unstarved conditions. This could also mean that *E. coli* response to the antibiotics used in our study is independent of the SR, although this is unlikely to be true, as work elsewhere contradicts this claim.

**Future Directions** The next step of characterizing the stringent-response-deficient mutants could be to perform swarming plate assays and assess the effect of valine-induced stringent condition on *E. coli* motility. It would also be beneficial to assess the effects of the stringent

response on biofilm formation and whether it is consistent with the antibiotic resistance pattern observed in the MIC assays. Additionally, since the *relA/spoT* double mutant unexpectedly displayed growth arrest in the nutrient rich condition that does not induce stringent response, future projects can look to ascertain the role that this pathway plays in *E. coli* under conditions that do not induce the stringent response.

## CONTRIBUTIONS

**MC** performed experiments and wrote, edited and revised the manuscript. **LL** performed experiments, edited and revised the manuscript. **AG** performed experiments, wrote and edited the manuscript. **NL** performed experiments, wrote and edited the manuscript.

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