Ampicillin Resistance Is Increased in *Escherichia coli* K12 relA and spoT Mutants but Sub-inhibitory Pretreatment Does Not Induce Adaptive Resistance

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The genes *relA* and *spoT* of *Escherichia coli* are required for the production of guanosine tetraphosphate, a critical molecule for the adoption of a stringent state that minimizes harm during metabolic and environmental stress. Sub-inhibitory treatments of antibiotics have been shown to result in subsequent antibiotic resistance, an effect also seen after stringency is induced through amino acid starvation. A *relA* mutant has been shown to lose this acquired resistance, suggesting a role for the stringent response in antibiotic resistance following sublethal exposure to the same antibiotic. This study examined an isogenic *ΔrelAΔspoT* strain to test the role of *relA* and *spoT* involvement in this possible mechanism of adaptive antibiotic resistance. Using minimal inhibitory concentration assays, wild-type, *ΔrelA* and *ΔrelAΔspoT* strains were grown in the presence of inhibitory ampicillin following a sub-inhibitory pretreatment of ampicillin, with growth rates measured through spectrophotometric analysis. These results demonstrate that pretreatment with sub-inhibitory levels of ampicillin does not induce resistance to subsequent inhibitory ampicillin concentrations in wild-type cells, while presence of *relA* appears to be decrease ampicillin resistance, possibly due to its role in peptidoglycan synthesis.

The stringent response in *Escherichia coli* mediates metabolic transcription in response to environmental and nutritional stress through regulating levels of the alarmone guanosine tetraphosphate (ppGpp) (1). Alarmones are intracellular mediators of stress that bind to RNA polymerase, altering promoter affinity in favour of transcripts that attempt to alleviate the metabolic strain, such as those involved in amino acid biosynthesis (2). Guanosinetetraphosphate synthesis is primarily regulated through the ppGpp synthetase gene *relA*, but is also inducible from the synthase corresponding to the *spoT* gene (3). Alone *spoT* is triggered by a greater range of environmental cues and is also involved in the turnover of ppGpp (3).

Although amino acid deprivation is the best described promoter of the stringent state (3), previous studies have implicated its induction through pretreating *E. coli* with sub-inhibitory levels of antibiotics (4). Sub-inhibitory treatments of kanamycin to *E. coli* B23 result in enhanced resistance to inhibitory levels of the same antibiotic, as well as other classes of antibiotics upon later exposure (5). Similarly, enhanced resistance to a broad range of antibiotics has been shown to result after induced stringency from amino acid starvation (6). Following a sub-inhibitory treatment of antibiotic, growth of a *ΔrelA* *E. coli* K12 mutant was significantly attenuated compared to the wild-type strain when grown in inhibitory concentrations of the same antibiotic (4). Taken together, these observations suggest an adaptive mechanism of antibiotic resistance after a sub-inhibitory exposure to kanamycin, mediated at least partially by the ppGpp synthetase product of *relA*. However, it is not clear if *relA* is directly involved in this resistance or contributes through indirect downstream processes.

Given the apparent lack of adaptive resistance to kanamycin in *ΔrelA*, it would be valuable to determine if other antibiotics can stimulate adaptive resistance through the stringent response, similar to what is seen during stringency following amino acid starvation (6). Utilizing a *ΔrelAΔspoT* strain completely removes ppGpp synthesis and allows testing of the involvement of the stringent response in adaptive antibiotic resistance. While a previous study has examined a *ΔrelAΔspoT* mutant, the site-directed mutagenesis approach used to create the double knockout generates unknown metabolic mutations (7). Furthermore, the study used a greater than sub-inhibitory concentration of antibiotic during the pretreatment, and focused on an *E. coli* B23 strain whereas the original observation of the *ΔrelA* strain was in K12 (4, 7).

To correct for possible genetic disturbances of the previously tested double knockout, an isogenic *ΔrelAΔspoT* K12 mutant, SL11W447-4, was constructed using the λ Red Site-Specific Recombinase System (8). Using *ΔrelA* and *ΔrelAΔspoT* strains, this report looks to demonstrate the role of the stringent response in adaptive antibiotic resistance. Furthermore, the growths of the strains were characterized in M9 media to facilitate future studies looking to induce stringency through amino acid starvation. Examining antibiotic resistance during secondary exposure in a *ΔrelAΔspoT* mutant is expected to reveal a greater attenuation in growth compared to wild-type and the *ΔrelA* strain, and should be reproducible if pretreatment with subinhibitory antibiotic is replaced by amino acid starvation.

**MATERIALS AND METHODS**

**Bacterial Strains.** Wild-type *E. coli* BW25113 (F-, ΔaraD-araB567, ΔlacZ4787::rmb-3), Δrph-1, Δ(rhaD-rhaB)568, hsdR514) and ΔrelAIW2755-3 (F-, Δ(araD-araB)567, ΔlacZ4787::rmb-3), Δrph-1, ΔrelA782::kan.rph-1, Δ(rhaD-rhaB)568, hsdR514) originate from the Coli Genetic Stock Center and were stored in the MICB 421

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TABLE 1 E. coli K12 strains utilized in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW25113</td>
<td>F−, ΔaraD-araA857, ΔlexA2789Δmbl-31, pCPP-1, hsdR514</td>
<td>CGSC #7636</td>
</tr>
<tr>
<td>JW2755-3</td>
<td>F−, ΔaraD-araA857, ΔlexA2789Δmbl-31, ΔaraG472, lacZΔM15-1, hsdR514</td>
<td>CGSC #10159</td>
</tr>
<tr>
<td>SL11W447-4</td>
<td>ΔrelA ΔspoT</td>
<td>Generated from JW2755-3</td>
</tr>
</tbody>
</table>

Laboratory stocks; ΔrelAΔspoTSL11W447-4 was previously generated using JW2755-3 as the parent strain (8).

Table conditions in Luria media. LB agar plates were made with and without antibiotic. The antibiotic plates were prepared using 100µg/ml ampicillin (Sigma #A9518) or 10µg/ml chloramphenicol (Sigma #C0378). Cultures were started by isolating colonies from cultured plates from the MCB 421 Laboratory Stocks and inoculated in LB media at 30°C in an air shaker. Except for the removal of chloramphenicol resistance, all experiments used an overnight culture that was grown in LB media at 30°C in an air shaker; liquid culture started from existing inoculated media was diluted 1/100.

Growth of SL11W447-4 in M-9 media. M-9 minimal media was prepared as previously described (7) with 0.5 mg/ml NaCl, 7.0 mg/ml NaHPO4, 3.0 mg/ml KH2PO4, 1.0 mg/ml NH4Cl, 0.2 mg/ml MgSO4 and 0.2% glycerol in dH2O. SL11W447-4 was grown in LB media overnight and then inoculated (1/100) in M-9 and grown for 48 hours at 30°C in an air shaker until growth was >1.0OD600.

Preparing electrocompetent SL11W447-4. Removal of the chloramphenicol resistance cassette inserted by the λ Red Site-Specific Recombinase System in strain SL11W447-4 is required for growth in minimal media. Overnight culture was inoculated 1/100 into LB and allowed to grow to an OD600 of 0.5 at 30°C. Cells were pelleted and washed with ice cold dH2O after the first two spins and 10% glycerol in dH2O after the third. The cells were resuspended in ice-cold dH2O.

Electroporation of SL11W447-4. 400ng of the pCP20 plasmid carrying FLP recombinase was added to 60ul of the cell suspension in a cuvette and electroporated at 2.49kV with a BioRad GenePulser (Bio-Rad #165-2105). The cells were added to SOC media as previously described (8) and allowed to recover for one hour at 30°C. The recovered culture was plated on 100µg/ml ampicillin LB agar plates at 30°C to select for transformants. Selected transformants were then plated on LB media and grown at 42°C to activate the FLP recombinase, and finally grown at 30°C on LB plates containing 100µg/ml ampicillin, 10µg/ml chloramphenicol or no antibiotic to confirm loss of resistance.

Ampicillin minimal inhibitory concentration (MIC) assay. Overnight cultures of each strain grown in LB media were diluted to an OD600 of 0.2. Each strain was transferred into a well plate containing final concentrations of ampicillin from 0.125µg/ml doubling up to 16ug/ml. The plate was allowed to grow at 30°C for 24 hours, and the MIC was reported by examining the lowest concentration of ampicillin that prevented growth individually for each strain, as reported in Table 2.

Antibiotic subinhibitory pretreatment. Overnight cultures of each strain were diluted in LB media to an OD600 of 0.1, treated with 0.25x the MIC of ampicillin as previously described (7), and incubated for one hour at 30°C in a shaking air bath.

Secondary exposure and growth assay. Pretreated cultures were given 1X the MIC of ampicillin and placed at 30°C in a shaking air bath. OD600 readings were taken every 25 minutes for 125 minutes with a Spectronic 20 spectrophotometer. A final reading was collected after 24 hours of growth.

RESULTS

Minimal Inhibitory Concentration Assay. Discovering the lowest concentration of antibiotic that inhibits growth in each strain is required to establish the sub-lethal pretreatment. As the ΔrelA parent strain utilized (JW2755-3) is kanamycin resistant, another antibiotic was needed to possibly induce stringency (8). Several antibiotics were tested (data not shown), however ampicillin was selected as all tested strains were susceptible within the range of concentrations used. The MIC assay illustrates that the elimination of relA halved the MIC while removing both relA and spoT reduced the MIC 8-fold from the wild-type (table 2). This conflicts previous studies, which have shown both an increase in the MIC of ampicillin on different ΔrelAΔspoT strains (7) as well as no effect compared to the wild-type (4, 9). While conflicting, this result follows the expectation that if stringency promotes increased antibiotic resistance, then removal of stringent response genes relA and spoT should reduce resistance.

Sub-inhibitory ampicillin does not induce resistance to subsequent inhibitory concentrations in wild-type. Figure 1a demonstrates the growth of wild-type strain BW25113 over 125 minutes. Most strikingly, cultures receiving 1x the MIC following the sub-inhibitory pretreatment grew equally well as those receiving 1x MIC after no pretreatment. Furthermore, cultures receiving just the pretreatment with no subsequent ampicillin exposure grew significantly more than the other conditions. This difference in growth is most prominent after 125 minutes but is also clear after 24 hours (Fig. 2a). This difference in growth suggests that sublethal levels of ampicillin does not stimulate adaptive antibiotic resistance, as it would be expected that pretreatment followed by 1x MIC would result in greater growth than a culture receiving 1x MIC without the previous antibiotic conditioning.

Removal of relA and spoT eliminates disparity in growth between treatments. Figure 1b and 1c demonstrate growth over 125 minutes of the ΔrelA and ΔrelAΔspoT strains, respectively. Unlike the wild-type culture, all three conditions grow equally well for both ΔrelA and ΔrelAΔspoT, even after 24 hours (Fig 2a,b). It is expected that removing the ppGpp synthesis genes would
FIG 1 Effect of subinhibitory ampicillin pretreatment on growth in inhibitory levels of ampicillin in (a) WT (b) ΔrelA and (c) ΔrelAΔspoT E. coli. (♦) is subinhibitory pretreatment followed by 1x MIC of ampicillin (■) is subinhibitory pretreatment followed by no further ampicillin and (▲) is no pretreatment followed by 1x MIC of ampicillin.

eliminate a potential mechanism for adaptive ampicillin resistance after a sub-inhibitory exposure. This expectation predicts that the pretreated mutants receiving 1x MIC would grow relatively similar to cultures receiving inhibitory ampicillin without the pretreatment, and that both conditions would have less growth than a culture just receiving sub-lethal ampicillin. It is further expected that this effect would be more pronounced in ΔrelAΔspoT than ΔrelA if the stringency machinery is responsible for adaptive ampicillin resistance. Instead, each condition grew equally well, a trend seen in both ΔrelA and ΔrelAΔspoT. While growth of ΔrelAΔspoT is significantly less than the other two strains over 125 minutes (Fig. 1), it greatly expands over 24 hours and results in similar differential cell growth to ΔrelA (Fig. 2).

FIG 2 Increase of growth of E. coli K12 WT strain compared to ΔrelAΔspoT and ΔrelA mutants after (a) 125 minutes and (b) 24 hours. Bars represent the first OD₆₀₀ reading subtracted from the final. White bars indicate sublethal pretreatment of 0.25x the MIC of ampicillin followed by the full inhibitory treatment; dark bars represent pretreatment with no full treatment while grey bars are no pretreatment followed by the full inhibitory treatment.

The relA/spoT mutant and growth in M-9 media. ΔrelAΔspoT is unable to grow from a plated culture directly to M-9 media. When the M-9 media was supplemented with casamino acids, amino acids, or a combination of both growth was negligible (data not shown). When the ΔrelAΔspoT mutant was first grown in LB and then transferred to M-9, growth was observed to be 0.047 OD₆₀₀ at 24 hours and 1.15 OD₆₀₀ at 48 hours compared to 1.860 OD₆₀₀ after 24 hours of growth in LB (Fig. 3). As expected, once growth was established in M-9 media subsequent inoculants of M-9 grew much faster than previously, reaching 0.89 OD₆₀₀ after 24 hours. When ΔrelAΔspoT from M-9 was used to inoculate LB the OD was 1.95 OD₆₀₀ after 24 hours and when reinoculated into M-9 the initial lag phase was absent and reached 0.648 OD₆₀₀ after 24 hours.

DISCUSSION
Numerous reports have conflictingly reported that a kanamycin pretreatment resulted in resistance to other aminoglycosides and antibiotics of different classes like ampicillin, resistance to only the same class or no resistance at all (4-5, 7, 9-10). As the ΔrelA/JW2755-3 strain utilized is kanamycin resistant, we focused on an antibiotic putatively linked to adaptive resistance to give insight into broad-spectrum resistance (4-5). Kohanski et.
al demonstrated that inoculating wild-type *E. coli* in the presence of sublethal ampicillin granted subsequent ampicillin growth, although the study utilized very minor levels of the antibiotic over a course of five days (13). Choosing ampicillin allowed testing if sublethal levels of antibiotics other than kanamycin were sufficient in inducing resistance, and if relA and spoT contributed to this possible mechanism.

The MIC assay (table 2) suggested an immediate role for *relA* and *spoT* in ampicillin resistance, with *ΔrelAΔspoT* being more susceptible to the antibiotic than *ΔrelA* or the wild-type. While this trend contrasts some earlier reports (4-5, 7) it is consistent with the work of Greenway et. al which examines the susceptibility of *E. coli* to a range of antimicrobial agents after removal of *relA* (6). As the synthesis of ppGpp leads to the regulation of a great diversity of metabolic gene activities (3), it is reasonable that removing *relA* and *spoT* would negatively impact the intrinsic defenses of the cell and its ability to grow. Indeed, both mutants saw less absolute growth than the wild-type over 125 minutes (Fig. 1).

Unexpected however, was the complete lack of adapted resistance, short or long term, seen in wild-type BW25113 after ampicillin pretreatment. (Figs. 1a, 2a). It is possible that, unlike kanamycin, ampicillin is unable to simulate stringent conditions and therefore no resulting antibiotic resistance is obtained. Kanamycin is an aminoglycoside antibiotic that interferes with translation by binding the 30S ribosomal subunit (11) while ampicillin belongs to the β-lactam family of antibiotics that interfere with the synthesis of peptidoglycan needed for the bacterial cell wall (12). The stress kanamycin places on protein production may emulate an environment deficient in amino acids, a well-known inducer of stringency (3).

Interestingly, the growth inhibition caused by 1x MIC administration of ampicillin to BW25113 wild-type cells was ablated in the *ΔrelA* and *ΔrelAΔspoT* strains at both 125 minutes and 24 hours of incubation (Figs. 2a, 2b). In these strains, the turbidity readings between the pre and non-pretreated groups exposed to inhibitory concentrations of ampicillin was comparable to the control group receiving no lethal treatment. This could be a consequence of the *relA* deletion in these two strains. The effect of ampicillin on the induction of bacterial cell death occurs in two phases: a “priming stage” and a “lysis induction” stage (14, 15). The priming stage is ampicillin dependent, whereby the β-lactam interacts with the penicillin binding proteins. Lysis induction is independent of ampicillin, and requires interference with peptidoglycan hydrolase activity which is followed by bacterial lysis (15). *relA* has been shown to exert control over peptidoglycan hydrolase activity (14); during the stringent response, ppGpp inhibits phospholipid and peptidoglycan synthesis (15, 16).

An absence of *relA* may thus interfere with bacterial lysis upon lethal ampicillin assault. This may explain why 1x MIC administration of ampicillin was far more effective at inhibiting bacterial growth in the wild-type strain compared to the *ΔrelA* and *ΔrelAΔspoT* strains. Though the induction of cell lysis may be compromised in the absence of *relA*, ampicillin is still able to bind target proteins during the ‘priming stage’ and inhibit growth. Indeed, one study observed that peptidoglycan hydrolase activity was required for penicillin to cause cell lysis and death, but would otherwise only inhibit cell growth (17).

The similarity in turbidity readings between the 1x MIC ampicillin treatments and the sublethal only pretreatment for the *ΔrelA* and *ΔrelAΔspoT* strains could be a secondary effect due to the ‘stalling’ of the cell at the priming stage of ampicillin induced-death. It is plausible that such an arrest could induce a cellular stress response, such as massive cell swelling that could cause an increase in the OD readings to levels comparable to growth without the antibiotic. Koch previously demonstrated that increasing the osmotic pressure placed on *E. coli* resulted in morphological changes that increased the colony’s turbidity (22). Similarly, Abram and Gibbons have shown that salts, detergents and heat altered the cell shape of *Halobacterium cutirubrum*, affecting the turbidity readings (23) Monitoring the cellular morphology of *ΔrelA* and wild-type strains following ampicillin treatment could help elucidate this possibility, as discussed in future directions.

We also started characterizing the growth of SL11W447-4 in M-9 minimal media to assist future studies inducing stringency through nutrient starvation. *ΔrelAΔspoT* strains have been observed to be auxotrophic for up to 10 amino acids (18). Contrary to previous reports, it was possible to grow SL11W447-4 in M-9 medium without supplementation. Inoculation from a loopful of culture from an LB plate did not result in growth of the *ΔrelAΔspoT* strain in M-9 media. However, when grown first in LB medium and then transferred to M-9, growth was achieved (Fig. 3). Although it is plausible that vitamin contamination is occurring from the transfer of LB liquid culture into M-9 media, sequential inoculations of M-9 that eliminate trace LB elements results in almost 20x greater growth in 24 hours and does not exhibit a lag phase when compared to primary growth in M-9. Also contrary to the first inoculation of M-9, when SL11W447-4 was transferred from M-9 to LB back to M-9 media the bacteria did not experience the initial lag phase that was observed (Fig. 3).
The initial lag seen in primary M-9 growth from LB can be attributed to the activation of required genes and signaling pathways to adapt to the minimal media (19). One study demonstrated that ΔrelAΔspoT double mutants had an increased rate of biofilm formation in LB when compared to growth in M9 minimal medium (20). Because growth in LB increases biofilm formation and biofilm formation is induced by ppGpp under stringent conditions (21), it is possible that biofilm formation could be essential for growth in M-9 and increasing biofilm formation, via growth in LB, aids the growth of ΔrelAΔspoT in M-9 media.

In conclusion, our report showed that ampicillin pretreatment does not result in adaptive resistance to the same antibiotic. However, we demonstrated that relA is involved in ampicillin resistance as ΔrelA and ΔrelAΔspoT had greater resistance than the wild-type strain. While the mechanism for this increased resistance is unknown, we speculate that it is a result of relA’s involvement in PG synthesis and that introduction of ampicillin to ΔrelA results in morphological changes to the ‘primed’ cell, increasing the turbidity. Finally, we were able to demonstrate that starting growth of SL11W447-4 in LB allowed growth in M-9 media, possibly by stimulating metabolic pathways like biofilm formation.

FUTURE EXPERIMENTS
Growing E. coli in amino acid-deficient M-9 minimal media is a well-established means of inducing stringency. It would be valuable to grow cells in M-9 media and simultaneously try and induce stringency in one set of strains by an ampicillin pretreatment and amino acid deprivation to another, performing an RNA accumulation assay (or other means of identifying stringency) on both sets to determine the relative recruitment of stringency obtained by the ampicillin pretreatment, if it at all. This would allow a more confident assessment of the extent stringency is involved during ampicillin pretreatment for future antibiotic resistance experiments.

Subsequent antibiotic exposure should also include antibiotics other than ampicillin to reveal any patterns of resistance not seen in this report, as well as the effect ΔrelAΔspoT may have on this putative resistance. For example, it would be interesting to see if an ampicillin pretreatment results in kanamycin resistance, similar to how a kanamycin pretreatment or amino acid deprivation grants ampicillin resistance. This may demonstrate that adaptive antibiotic resistance is not universally promoted by antibiotics, but can be seen in kanamycin due to common downstream effects seen in stringency induced by nutrient starvation.

While adaptive antibiotic resistance has been examined in ΔrelA after exposure to sublethal kanamycin, it has not been examined in an isogenic ΔrelAΔspoT strain. Removing the kanamycin resistance cassette in JW2755-3 and repeating those experiments would help establish the extent to which relA and spoT individually contribute to adaptive resistance. Again, this data should be compared to strains that underwent amino acid starvation.

Finally, it would be valuable to image and characterize the physiology of all strains treated with ampicillin, kanamycin and amino acid starvation. This would help establish if cell swelling in the mutants is indeed impacting the turbidity readings. It is expected, based upon our results, that eliminating relA increases cell size upon ampicillin addition when compared to wild-type.

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