Journal of Experimental Microbiology and Immunology (JEMI) Vol. 15: 59 – 63

On the Limited Role of *relA* in Kanamycin and Amino Acid Starvation Induced Stringency and Subsequent Antibiotic Cross-protection in *Escherichia coli*

Victor Chiang, Benjamin Wong, Calvin Wong and Jaemin Yu
Department of Microbiology & Immunology, University of British Columbia

Two genes, *relA* and *spoT*, are known to be responsible for the production of ppGpp when *E. coli* cells undergoing amino acid starvation initiate the stringent response. In this modified state, cells have been reported to up-regulate processes which confer resistance to antibiotics. However, the individual contribution of *relA* was not separated from potential effects of *spoT*. By using 3 strains of *E. coli*: B23 (wild-type), 58-161 (*spoT*), and w1655 (*relA*, *spoT*) grown in modified M9 media, we attempted to characterize the impact of ppGpp synthase (RelA) in the induction of a stringent response, and whether there was a variation in stringency generated by exposure to kanamycin or amino acid starvation. Our results showed both treatments to have similar effects across all three *E. coli* strains, with no significant differences attributed to the particular genotypes. Furthermore, neither treatment appeared to confer antibiotic cross-protection upon subsequent exposure to sub-lethal concentrations of ampicillin.

In previous studies by Cheng *et al.*, antibiotic resistance of *Escherichia coli* has been attributed to the stringent response (2). When cells are starved of amino acids, tRNAs lacking attached amino acid may accumulate and bind to the A site of a ribosome, causing the ribosome to stall and activate a signaling cascade ultimately resulting in the production of guanosine-3',5'-bispyrophosphate (ppGpp) (17). This molecule specifically binds near the active site of RNA polymerase and halts the production of tRNA and rRNA (17) thereby resulting in an approximately three fold reduction in the rate of RNA synthesis (4). ppGpp also causes a physiological change at the cell surface (7,20) that might inhibit the uptake of certain antibiotics (2,5). ppGpp synthase I, encoded by the *relA* gene, is thought to be the initiator of this response (2).

By exposing the cells to sub-lethal levels of kanamycin, it was shown that cells were induced into a stringent response similar to that of amino acid starvation that exhibited increased resistance to subsequent antibiotics (2). When kanamycin is introduced to the cell, it binds to the 30S ribosomal subunit and prevents the migration of peptidyl tRNA from A-site to the P-site (1). During this inhibition, it is possible that the charged tRNA will leave the A site allowing a chance for uncharged tRNA to enter (2). In this condition, cells up-regulate the production of ppGpp, which is the behavior observed in cells undergoing amino acid starvation (2).

Cheng *et al.* used isogenic *E. coli* B 23 strains C78 (wild-type) and C79 (*relA*) in an attempt to determine the role of *relA* in stringent response induced by sub-inhibitory levels of kanamycin (2). These cells were subjected to sub-inhibitory levels of kanamycin as a pre-treatment and divided into two separate batches, one of which would be further treated with minimum inhibitory concentrations (MIC) of either ampicillin or tetracycline (2). Growth was measured directly using turbidity and indirectly using radioactive incorporation as measure of RNA synthesis. The *relA* mutant displayed lower survival rate during pre-treatment and lower resistance against subsequent exposure to ampicillin and tetracycline (2). However, RNA synthesis showed an increased trend for both strains that indicated RNA synthesis, which was not expected of C79 strain (2). This led to the prediction that other regulatory factors were responsible and the likely suspect was *spoT* (2,6). *spoT* was shown to encode a bi-functional ppGpp 3'-pyrophosphohydrolase that, in low pyrophosphate conditions, was capable of producing ppGpp in the absence of *relA* (20).

For this study, we sought to better characterize the role of RelA in stringent response induced by kanamycin treatment and compared the results with stringency elicited by amino acid starvation.

We expected several outcomes should RelA prove to have a significant contribution to the stringent response: wild-type and single mutant exposed to ampicillin after being starved of amino acid should exhibit higher survival rates than double mutant, in which *relA* is knocked out. RNA synthesis in double mutant should be higher than the other two strains as RNA synthesis was expected to continue in relaxed strains even after the cells were starved, while cells in stringency should

have impaired global RNA synthesis. If kanamycin induced a similar stringent response as in amino acid starvation when *relA* was present, similar results should have been observed. Conversely, if *relA* did not induce stringency that conferred antibiotic resistance, survival rates and radioactivity incorporation results should be similar between single and double mutant regardless of treatment.

MATERIALS AND METHODS

Bacterial Strains and preparation of overnight culture. *E. coli* strains 58-161 (F+, *bio-1*(Unst), *spoT1*, *metB1*, *creC510*) and w1655 (F+, *&lambda*$^-$, *relA1*, *spoT1*, *metB1*, *creC510*) were obtained from the Coli Genetic Stock Centre. *E. coli* B23 wild type strain was obtained from the MICB 421 culture stock collection in the Department of Microbiology and Immunology at the University of British Columbia. The 3 strains were grown at 37^{o}C in M9 minimal salt media modified with amino acid and biotin supplement.

Preparation of modified M9 minimal salt media. M9 minimal salt media was prepared as described preiously (9) with the following final concentrations (in the combined media): solution 1 was prepared with 0.5 mg/ml of NaCl (Fisher Scientifics), 7.0 mg/ml of Na_2HPO_4 (Sigma), 3.0 mg/ml of KH_2PO_4 (Fisher Scientifics), and 1.0 mg/ml of NH_4Cl (Fisher Scientifics), solution 2 was prepared with 0.2 mg/ml $MgSO_4 \cdot 7H_2O$ (Fisher Scientifics), and solution 3 was prepared with 3.5 mg/ml of glycerol (BDH B28454-76). The amino acid mixture was prepared with 2.0 mg/ml Bacto casamino acids (Difco DF0231172), 0.05 mg/ml glutamine (Sigma G-5763), 0.05 mg/ml asparagine (Sigma A-0884), and 0.01 mg/ml tryptophan (Sigma T-0254). The four solutions were autoclaved separately and combined, then 5N NaOH was added to adjust the pH to 7.2. 2 ml of L-biotin (Sigma B-4501) was autoclaved and added to the medium to accommodate the growth of *E. coli* 58-161 biotin auxotrophic mutant.

MIC assay. Overnight cultures of *E. coli* B23/58-161/w1655 were diluted in fresh modified M9 media to obtain a final turbidity of 0.01 OD_{460}. 100 µl of the appropriate diluted culture was pipetted into each well of two 96 well polycarbonate plates, such that each plate contained equivalent amounts of diluted culture of all 3 strains. 50 µl of fresh modified M9 media was added to make up a volume of 150µl in each well. 50 µl of kanamycin (Sigma K-4000) and ampicillin (Sigma A-9518) solution was added separately to each plate, such that one plate contained a starting kanamycin concentration of 250 µg/ml and the other contained a starting concentration of 125 µg/ml of ampicillin. Seven serial 1-in-4 dilutions were performed to give a final antibiotic concentration of 0.015 µg/ml for kanamycin and 0.003 µg/ml for ampicillin. 50 µl of the mixture was removed from the final row to keep the volume constant across every well. The plates were incubated at 37^{o}C for 48 hours and the MICs were determined by observing the well with the lowest concentration of antibiotic where bacterial growth was inhibited.

Preparation of Starvation Media. For starvation media the M9 minimal salt medium was supplemented with an amino acid mixture according to ratios listed in the Physiology of the Bacterial Cell (8) to give the following final concentrations: 0.1 mg/ml alanine (NBCo), 0.06 mg/ml arginine (Sigma A-5006), 0.05 mg/ml asparagine, 0.05 mg/ml aspartate (NBCo), 0.02 mg/ml cysteine (Sigma C-8755), 0.05 mg/ml glutamate (NBCo), 0.05 mg/ml glutamine, 0.12 mg/ml glycine (EMD GX0205-1), 0.02 mg/ml histidine (Sigma H-6034), 0.09 mg/ml leucine (Sigma L-8000), 0.07 mg/ml lysine (Difco 0705-11), 0.03 mg/ml methionine (NBCo), 0.04 mg/ml phenylalanine (Sigma P-5030), 0.04 mg/ml proline (Sigma P-0380), 0.04 mg/ml serine (Sigma S-4500), 0.05 mg/ml threonine (Sigma T-8625), 0.01 mg/ml tryptophan (Sigma T-0254), 0.03 mg/ml tyrosine (Sigma T-3754), and 0.08 mg/ml valine (Sigma V-0500). Isoleucine was excluded to induce starvation as previously described (17).

Induction of Stringent Response. Cells from overnight culture were diluted in modified M9 media to obtain an initial starting OD_{460} of 0.15-0.18 and incubated in a 27^{o}C shaking water bath for 50 minutes. In the kanamycin pretreatment, kanamycin was added to the inoculated culture to obtain 0.25 times the observed MIC. For amino acid starvation, 2ml of culture was inoculated in 18 ml of starvation media. In each of the 3 sets of treatments (control, kanamycin pretreatment, amino acid starvation), stock uracil (NBCo) was added to give a final concentration of 2 µg/ml and 6 ml of culture was transferred to separate test tubes for radioactivity incorporation assay before the culture was re-introduced into the water bath.

Turbidity Readings and Radioactivity Incorporation Assay. Starting at 5 minutes, turbidity readings were taken at OD_{460} for 60 minutes at 10 minute intervals using Spectronic 20 spectrophotometer. The radioactivity incorporation assay was performed alongside turbidity readings by adding 0.50µCi ^{14}C-uracil (Sigma), then transferring 50 µl of culture onto filter disks (Whatman 1003-323) in duplicate. The disks were washed in TCA and ethanol then incubated at 110^{o}C and tabulated with scintillation counts.

Determination of Ampicillin Cross Protection. After the initial set of turbidity readings, ampicillin was added to the culture at 0.25 times the MIC, and readings were continued for another hour. For the sets that underwent kanamycin pretreatment and amino acid starvation, 2 ml of culture was transferred to 8 ml of fresh modified M9 media before addition of ampicillin. Radioactivity incorporation was stopped before addition of ampicillin.

RESULTS

Minimum inhibitory concentrations of kanamycin determined for the wild-type, *spoT* and *relA, spoT* were not as expected. *relA* is known to be a key gene for the induction the stringent response, and as such we would have expected the wild-type *E. coli* B23 and the *spoT* single mutant to have lower MIC's than the *relA, spoT* double mutant as they should have been sensitive to kanamycin and would have entered into a stringent response, whereas the relaxed double mutant would lack such regulation and continue to grow in higher sub-lethal concentrations (2,3,4). The wild-type *E. coli* B23 was of a separate lineage than the *spoT* single mutant, and thus it would have been reasonable here to observe a slight difference between it and the single mutant's MIC's due to potentially many small underlying genetic differences (13). The MIC's observed for ampicillin were as expected. Beta-lactam antibiotics are known to be less effective on slow growing bacteria, which was in agreement with observations that our single and double mutant strains always grew at a notably slower rate than the wild-type

TABLE 1. Minimum inhibitory concentrations for *E. coli* wild-type, single mutant, and double mutant strains.

Antibiotic	Minimum Inhibitory Concentration (µg/ml)		
	wild-type	*spoT*	*relA/ spoT*
Kanamycin	16	4	4
Ampicillin	8	31	31

TABLE 2. Effect of *spoT* and *relA/spoT* mutations on resistance to ampicillin after treatment of the strains by kanamycin or isoleucine starvation.

Treatment	Growth rate (10^{-3})					
	Wild-type		*spoT*		*relA/spoT*	
	Before ampicillin	After ampicillin	Before ampicillin	After ampicillin	Before ampicillin	After ampicillin
Control	6.5	2.7	4.1	3.9	5.0	3.7
Kanamycin	0.4	-0.2	0.2	-0.3	0.1	-0.4
Starvation	1.2	0.9	1.0	0.5	0.7	0.4

during the course of our experiments (Table 2) (18).

Growth rates observed during pretreatment and responses to subsequent ampicillin exposure were not as expected. Under kanamycin and isoleucine starvation conditions we would have expected the *relA* double mutant to have the highest growth rate due to its supposed inability to enter into the stringent response (2,3,4). Likewise, we expected the wild-type and single mutant kanamycin and isoleucine pretreated cultures to respond in similar fashion upon exposure to ampicillin, but comparative growth rates for all strains before and after exposure clearly indicate a greater detrimental effect in the kanamycin treated cultures.

DISCUSSION

In most aspects of our experiment, the *E. coli* test strains did not behave as expected. In general, it was expected that our wild-type and *spoT* single mutant strains should have been more sensitive than the *relA*, *spoT* double mutant to exposure to kanamycin or isoleucine starvation, as these strains retained the *relA* gene and by extension the ability produce ppGpp, the central alarmone in the induction of the stringent response (20). For the double mutant lacking *relA* we would have expected to observe higher growth rates under the aforementioned stress conditions. This was not the case however, as we first saw in our kanamycin MIC assay that the double mutant had the same MIC as the isogenic single mutant and a four-fold lower MIC than the wild-type (Table 1). This may have been an inherent problem with the design of our assay in that the four-fold dilution increments were rather large. The double mutant may have been able to grow in twice the MIC of the single mutant for example, but our increment sizes would not have allowed us to see that. Alternatively, aminoglycosides are known to be more effective against fast growing bacteria, so the low MIC could have actually been a reflection of a higher growth rate (19).

In any case , none of the determined MIC values differed by more than one dilution increment between the three strains, which meant that within an error range of one increment, the actual MIC values could have been the same. This also introduces the possibility that our chosen treatment concentrations of 0.25 times the MIC were actually been too low to achieve the desired effects. This seemed quite plausible upon review of our growth rate data (Table 2) where in both our control and starvation test sets we observed only a minor decreases in the growth rates of all our strains after the addition of 0.25 times the MIC of ampicillin, an indication that the antibiotic was not present in high enough a concentration to interfere with peptidoglycan synthesis in a significant portion of the cell culture populations. Unfortunately, because we performed the control set first and the starvation set last, this trend did not become obvious until after we had completed all our experiments.

While the sharp declines after ampicillin exposure seen in our kanamycin treatment set would appear to suggest that perhaps we did use enough antibiotic, this was most likely an artifact of our experimental design. Our method for the kanamycin treatment set involved growing an overnight culture and then using it to inoculate a flask of fresh media containing 0.25 times the MIC of kanamycin. This dilution meant that our inoculum would not have grown to a high enough density during the initial hour of pretreatment. Then at the end of the first hour, the culture was diluted again, this time into fresh media containing 0.25 times the MIC of ampicillin. There were two major flaws with this design. Firstly, performing the second dilution into media lacking kanamycin would have essentially had the effect of relaxing our cells and undoing the pretreatment, rendering the subsequent ampicillin cross-protection assay pointless. Secondly the large amount of dilution would have made the culture more vulnerable to ampicillin simply due to kinetics.

At least for antibiotics that bind to cellular components, like ampicillin binds to penicillin binding proteins (11), denser cultures should have a naturally higher survivability because the number of cells would have a diluting effect, physically limiting the maximum number of antibiotic molecules per cell (16). As long as the cellular concentration of ampicillin remains sub-lethal, cells can eventually recover by replacing their

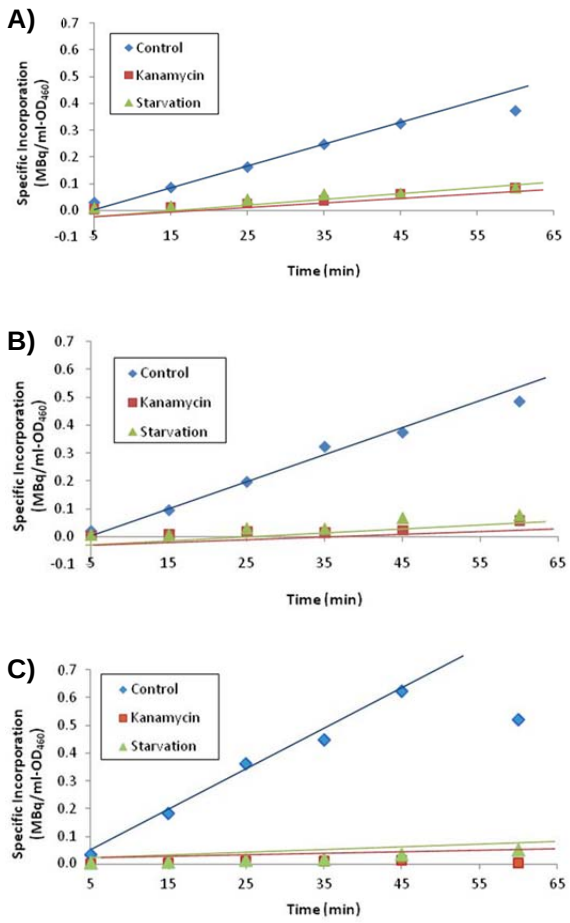

FIG. 1. Comparative RNA synthesis per cell under control, kanamycin, and isoleucine starvation treatments for: A) wild-type, B) single mutant, and C) double mutant strains. As an alternate measure of growth during our pretreatments, the degrees of RNA synthesis per cell measured by ^{14}C-uracil radioactive incorporation strongly mirror the relative rates of growth seen in Table 2., and suggest the same problems as previously discussed.

defective penicillin binding proteins with new ones under favorable growth conditions (14). Since our twice diluted kanamycin treatment culture would have been relaxed and had a low cell density, it was no surprise that the growth rate dropped so notably upon ampicillin exposure. In short, key flaws in our experimental design have made it impossible compare any potential cross protection conferred by kanamycin induced stringency to that induced by starvation.

When we examined only the pretreatment growth rates, we noticed that contrary to our expectations, the *relA*, *spoT* double mutant did not have the most rapid growth while under exposure to kanamycin or isoleucine starvation, even though we expected it to since the absence of *relA* and *spoT* should have meant that it was in a constantly relaxed state. Instead, it was the wild-type that always had the highest growth rate. This was mostly likely due to the absence of the *spoT* gene in our single and double mutants. Apparently, even in the absence of *relA*, cells can express basal levels of ppGpp, so without *spoT* encoded hydrolase activity, both our single and double mutant stains would have accumulated ppGpp and would have consistently been in a semi stringent, slow growing state (11,15). We originally chose to use both single and double mutant strains in an attempt to better quantify the individual contribution of *relA* separately from the residual ppGpp synthetase activity of *spoT* (17). Ironically, the *spoT* mutations in our strains may have ended up acting as more of a fudge factor than a clarification.

As is, our results are inconclusive. We did not find support for a specific or significant role for *relA* in the induction of stringent responses, nor did we observe any comparability in responses when sub-lethal levels of kanamycin and isoleucine starvation were used as different modes of induction. Furthermore, these two methods of pre-treatment were not found to confer any protection against subsequent exposures to sub-lethal concentrations of ampicillin.

FUTURE EXPERIMENTS

We recommend several changes to our experimental method. For testing cross-protection, we recommend adding sub-lethal levels of the secondary antibiotic directly to the pre-treatment conditions, this will avoid any unnecessary dilution which could potentially influence the results of the subsequent cross-protection assay, as well as the relaxing effect which would undo the pre-treatments and also confound the results. Simpler changes include performing the initial MIC assays in smaller dilution increments to determine more accurate concentrations of antibiotics to use, and allowing cultures to grow to a higher starting turbidity such that only more significant growth changes would be noticeable in the overall trend.

ACKNOWLEDGEMENTS

The members of team 1α would like to thank the MICB 421 teaching staff for their counseling and guidance. We would also like to thank the media room staff and the microbiology department of UBC for providing advice and necessities required for our experiments.

REFERENCES

1. **Chen, L. X., S. He, C. Li, and J. Ryu.** 2009. Sublethal kanamycin induced cross resistance to functionally and structurally unrelated antibiotics. J. Exp. Microbiol. Immunol. **13:** 53-57.
2. **Cheng, K., L. Ren, and K. Tsai.** 2010. Inhibitory concentrations of Kanamycin in the presence of ppGpp Synthase RelA protection against subsequent lethal antibiotic assaults in *E. coli* CP78. J. Exp. Microbiol. Immunol. **14:** 51-56.

3. **Davis, B. D.** 1987. Mechanism of bactericidal action of aminoglycosides. Microbiol. Revs. **51:** 341-350.
4. **Gallant, J.** 1979. Stringent Control in E. coli. Ann. Rev. Gen. **13:**393-415.
5. **Gilbert, P., P. J. Collier, and M. R. W. Brown.** 1990. Influence of growth rate on susceptibility and antimicrobial agents: biofilms, cell cycle, dormancy, and stringent response. Antimicrob. Agents Chemo. **34:** 1865-1868.
6. **Hernandez, V. J., and H. Bremer.** 1991. *Escherichia coli* ppGpp Synthetase II activity requires *spoT*. J. Biol. Chem. **266:** 5991-5999.
7. **Mittenhuber, G.** 2001. Comparative genomics and evolution of genes encoding bacterial (p)ppGpp synthetases/hydrolases (the Rel, RelA and SpoT proteins). J. Mol. Microbial. Biotechnol. **3:** 585-600.
8. **Neidhardt, F. C., J. L. Ingraham, and M. Schaechter**. 1990. Physiology of the bacterial cell. A molecular approach. Sinauer associates.
9. **Neidhardt, F. C., P. L. Bloch, and D. F. Smith.** 1974. Culture medium for enterobacteria. J. Bacteriol. **119:** 736 – 747.
10. **Nierlich, D. P.** 1978. Regulation of bacterial growth, RNA, and protein synthesis. Ann. Rev. Microbiol. **32:** 393 – 432.
11. **Presslitz, J.E.** 1978. Mode of action of a structurally novel beta-lactam. Antimicrob. Agents Chemo. **14:** 144-150.
12. **Ramey, W. D. (ed.).** 2010. Microbiology 421: Manual of experimental microbiology. University of British Columbia: Department of Microbiology and Immunology.
13. **Rod, M. L., K. Y. Alam, P. R. Cunningham, and D. P. Clark.** 1988. Accumulation of trehalose by *Escherichia coli* K-12 at high osmotic pressure depends on the presence of amber suppressors. J. Bacteriol. **170:** 3601 – 3610.
14. **Rodionov, D.G., and E.E. Ishiguro.** 1996. Effects of inhibitors of protein synthesis on lysis of *Escherichia coli* induced by β-lactam antibiotics. Antimicrob. Agents Chemo. **40:** 899-903.
15. **Sarubbi, E., K. E. Rudd, H. Xiao, K. Ikehara, M. Kalman, and M. Cashel.** 1988. Characterization of the *spoT* gene of *Escherichia coli*. J. Biol. Chem. **264:** 15074-15082.
16. **Sieradzki, K., and A. Tomasz.** 2006. Inhibition of the autolytic system by vancomycin causes mimicry of vancomycin-intermediate *Staphylococcus aureus*-type resistance, cell concentration dependence of the MIC, and antibiotic tolerance in vancomycin-susceptible *S. aureus*. Antimicrob. Agents Chemother. **50:** 527 – 533.
17. **Traxler, M. F., S. M. Summers, H. T. Nguyen, V. M. Zacharia, G. A. Hightower, J. T. Smith, and T. Conway.** 2008. The global, ppGpp-mediated stringent response to aminoacid starvation in *Escherichia coli*. Mol. Microbiol. **68:** 1128-1148.
18. **Tuomanen, E., R. Cozens, W. Tosch, O. Zak, and A. Tomasz.** 1986. The rate of killing of *Escherichia coli* by β-lactam antibiotics is strictly proportional to the rate of bacterial growth. J. Gen. Micbiol. **132:** 1297 – 1304.
19. **Watanabe, T.** 1959. Cross resistance of *Salmonella typhimurium* to streptomycin and kanamycin. Keio University School of Medicine: Department of bacteriology. **78:** 148 – 149.
20. **Xiao, H., M. Kalman, K. Ikehara, S. Zemel, G. Glaser, and M. Cashel.** 1991. Residual guanosine 3',5'-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. J. Biol. Chem. **266:** 5980-5990.