Journal of Experimental Microbiology and Immunology (JEMI) Vol. 15: 52 – 58

Assessing the Role of SpoT and RelA in Capsular Polysaccharide Synthesis after Treatment with Sub-lethal Concentrations of Kanamycin to Confer Decreased Antibiotic Sensitivity in *Escherichia coli*

Wesley Chenne, Louisa Ng, and Mary Rose Pambid
Department of Microbiology & Immunology, University of British Columbia

Resistance to various antibiotics has been associated with increased capsular polysaccharide production through activation of RelA and SpoT, regulators of the stringent response, through their respective synthesis and hydrolysis of guanosine tetraphosphate in *Escherichia coli*. In order to further characterize the role of SpoT and RelA in capsular polysaccharide production and in conferring antibiotic resistance, growth patterns and induced capsular polysaccharide levels of various strains were determined following sub-lethal treatment with kanamycin and further treatment at inhibitory levels of kanamycin. Contrary to previous reports that capsular polysaccharide confers antibiotic resistance, it was found that *spoT* mutants produced the least capsular polysaccharide but had higher levels of resistance in comparison to *relA* mutants, which produced the most polysaccharide but exhibited lower resistance. As expected, both these mutants exhibited lower resistance to kanamycin as a result of pre-treatment than the wild-type strain. Thus, we propose that bacterial survival and resistance development upon antibiotic administration is dependent upon a myriad of factors that stem from the regulation of guanosine tetraphosphate which not only include capsular polysaccharide levels, but also other pathways leading to and resulting from the induction of the stringent response.

For *Escherichia coli*, a general response to environmental stressors, such as nutrient starvation and the administration of antibiotics, is the induction of the stringent response (16), which promotes cell survival at the expense of exponential cellular growth. The stringent response is regulated by the expression of the *rpoS* gene and its end product, sigma factor s (σ^S) (6, 11). This expression is upregulated by the activity of the alarmone guanosine tetraphosphate (ppGpp) in conjunction with *dskA* (15, 21). In turn, the two major cellular factors that have been implicated in the production of ppGpp are SpoT and RelA (21). When cells are treated with aminoglycosides such as kanamycin (KAN), its activity leads to the simultaneous upregulation of both RelA and SpoT activity (1). KAN binds to the 30S subunit of the ribosome, leading to blockage of the anti-codon/codon binding site and limitation of protein synthesis (3, 10). RelA binds to these blocked ribosomes and mediates the conversion of ATP and GTP to AMP and ppGpp (19). Moreover, KAN also causes membrane damage and a decrease in intracellular ferrous iron via the induction of the bacteriocidal Fenton hydroxyl radical reactions (10), which induces SpoT activity and the production of ppGpp (18) However, SpoT is not only implicated in the production of ppGpp, but primarily serves as the hydrolase for the degradation of ppGpp to ensure that cell homeostasis can be achieved upon return to normal environmental conditions, as the decrease in ppGpp results in downregulation of *rpoS* and a reduction in the expression of σ^S (14).

Upon exposure to sub-lethal levels of KAN or other aminoglycosides, a downstream effect that has been observed is the induction and up-regulation of capsular polysaccharides (CPS) (1). CPS has been shown to confer protective effects for *E. coli* by putatively limiting the entry and activity of KAN and other aminoglycosides (20). Recent studies with *relA* mutants have been performed with regards to the effects of RelA on conferring lessened antibiotic sensitivity after pre-treatment with sublethal levels of KAN, but they have shown inconclusive evidence for its exact role (1). Moreover, the role of SpoT in the relationship between conferring lessened antibiotic sensitivity and the production of CPS upon sub-lethal treatments of KAN requires more characterization.

We hypothesized that upon pre-treatment with sub-lethal levels of KAN, the presence of both SpoT and RelA would confer the greatest decrease in antibiotic sensitivity in a parental wildtype strain, followed by

relA, *spoT*, and *relA/spoT* mutant strains. To study the effects of these gene products, isogenic mutant strains were assayed for initial sensitivity to KAN, then pre-treated with sub-lethal levels of the antibiotic prior to conducting measures of growth at different inhibitory concentrations. To further elaborate the role of CPS in conferring a decrease in antibiotic sensitivity, the induced amounts of CPS were also qualitatively and quantitatively assayed following this initial sub-lethal pre-treatment with KAN. In comparison to *relA* mutants, *spoT* mutants were shown to have higher resistance despite exhibiting lower levels of CPS production. In fact, the *relA* mutant was shown to produce the highest levels of CPS, yet its resistance fell short of both the *spoT* and the wild type strain. Finally, the *spoT/relA* double mutant displayed slightly lower CPS production than the wild-type, but had the lowest levels of resistance; this suggests that other than the induction of CPS due to *spoT* and *relA*, there are also other cellular processes that result from these two regulators involved in the induction of antibiotic resistance.

MATERIALS AND METHODS

Bacterial strain. *E. coli* strains WG1 (WT), AT-2(*relA*), 58-161(*spoT*), AB301 (*relA, spoT*) as described in Table 1 were obtained from the Coli Genetic Stock Centre, Yale University.

Preparation of M9 minimal salts media supplemented with vitamins and amino acids. M9 minimal media (1) was prepared with 0.4% glycerol as the carbon source. A amino acid supplement was prepared by adding 0.6 g of Casamino Acids (Bacto #223030) in 200 ml of distilled water, 0.6 g of L-tryptophan (Sigma #T0254), 0.6 g of L-glutamine (Sigma #G5763), and 0.6 g of L-asparagine (Difco 0583-12) in 200 ml of distilled water. Biotin solution was prepared by adding 20 mg of D-biotin (Sigma #B4501) to 120 ml of distilled H_2O and filter sterilized using a 45 um filter. Biotin was added to a final concentration of 10 µg/ml.

Overnight (ON) cultures. Overnight (ON) cultures of each *E. coli* strain were prepared by inoculating 10 ml of supplemented M9 minimal salts media with a loopful of bacteria and incubating in an air shaker with mild aeration (50-100 rpm) at 37°C.

Minimum Inhibitory Concentration (MIC) assay. ON cultures were diluted by a factor of 1000, and 100 ul of each diluted strain was added to the wells of two rows of a 96 well plate. Stock KAN solution at 20 mg/ml was diluted to 1 mg/ml, and 100 µl was added to each well of the first column. Serial half dilutions were performed across the rows by transferring 100 µl from the wells in the first column to the wells of the next column, leaving the last column without KAN. Following incubation for 24 hours on a shaking platform at 37°C, the MIC for each strain was defined as the wells with the lowest concentration of KAN at which there was no bacterial growth.

Sub-lethal antibiotic pre-treatment. ON cultures of each strain were used to inoculate a 1-in-20 dilution of M9 media supplemented with amino acids and vitamins. All the cultures were incubated for 3 hours at 37°C in a shaking water bath prior to addition of KAN. Based on the results of the MIC assay, KAN solution was diluted to 0.25X MIC for each strain as described in Table 2 and added to each flask. All the flasks were then incubated for another hour. Each strain was also diluted and incubated similar to the pre-treated cultures in separate flasks without adding KAN to create untreated cultures as controls.

TABLE 1. Bacterial strains acquired from *E. coli* Genetic Stock Center.

Strain	Genotype
WG1 (WT)	F+, *ΔrfbB51*, F1-1
AT-2 (*relA*)	Hfr(PO22), *relA1*, *metB1*
58-161 (*spoT*)	F+, *bio-1*(Unst), *spoT1*, *metB1*, *creC510*
AB301 (*relA/spoT*)	Hfr(PO21), *relA1*, *spoT1*, *metB1*

Antibiotic sensitivity test. The sensitivity of each strain of *E. coli* to KAN was tested by growing each strain at four different levels of antibiotic based on the MIC of the strain. For each strain, four different flasks containing 90 ml of M9 minimal salts media were inoculated with 10 ml of each pretreated culture. KAN was added at concentrations of 1 MIC, 2 MIC, and 3 MIC to three of the flasks; the last flask was not subjected to KAN treatment. After measuring and recording the initial turbidity of the cultures, the bacteria were incubated at 37°C in a shaking water bath, and further turbidity measurements were taken and recorded at 15, 30, 60, 100, and 160 min. following the first measurement. The cultures were grown overnight and the final turbidity was read and recorded. All turbidity readings were performed using a Spectronic 20D spectrophotometer with wavelength set at 460 nm, using M9 minimal salts media as a blank.

CPS staining. *E. coli* capsules were stained with Congo Red and Maneval's solution (2) by preparing air dried and heat fixed slides of pretreated and untreated cells on glass slides. Slides were visualized by light microscopy at 1000x magnification. Images were acquired by aligning a Samsung ST50 digital camera with the ocular lens at ISO 100, f/56, and exposure time of 1/28 seconds.

Capsule isolation. CPS was isolated by following a modified version of the methods outlined by Lu *et a* (10). 60 ml of each pretreated and untreated bacterial cultures were centrifuged at 17,000 x g for 20 min. The supernatants were discarded and the pellets were resuspended in 40 ml of PBS (137 mM NaCl, 2.7 mM KCl 1.4mM Na_2HPO_4, 1.8 mM KH_2PO_4). Following addition of ice cold acetone, samples were centrifuged at 6,000 x g for 10 min. After discarding the supernatants and resuspending the pellets, the samples were transferred into 1 ml/cm Spectra/Por® molecularporous membrane dialysis tubes with molecular weight cut-offs at 6,000 – 8,000 kDa. The samples were dialyzed against 2 litres of distilled water at 4°C for 48 hours, after which a lyophilizer was used to dry the isolated expolymers overnight. Each dried sample of expolymers was dissolved in 3 ml of 10 mM $MgCl_2$. Ribonuclease A (Fermentas #EN0531) and Deoxyribonuclease I (Sigma #2326670) were added to the dissolved samples, which were incubated for 3 hours at 37°C in a shaking water bath. Pronase (Boehringer Mannheim #165921) was added to the samples, which were incubated at 37°C in a shaking water bath overnight. To purify the exopolymers, samples were

TABLE 2. Sub-lethal (0.25X MIC) concentrations of kanamycin used in pre-treatment

Strain	Kanamycin (µg/mL)
WT	3.9
relA	0.24
spoT	1.95
relA/spoT	0.98

TABLE 3. Minimum inhibitory concentrations of kanamycin for *E. coli* strains.

	Strains			
	WT	*relA*	*spoT*	*relA/spoT*
MIC (μg/ml)	16	4	8	1

treated as described by Lu *et al*. (10), and dialysis and lyophilization were performed as previously described. All centrifugations were performed using a Beckman J2-21 centrifuge with a JA-20 rotor.

Anthrone carbohydrate assay (10). Anthrone reagent was prepared as described by Lu *et al.* (10). A glucose standard was prepared using a stock solution of α-D(+) glucose (Sigma, #G-5000) and M9 without glycerol at a concentration of 364 μg/L, and serially diluting the solution to concentrations of 364, 182, 91, 45.5, 22.25, 11.25, and 5.5 μg/ml, while leaving one tube of M9 without glycerol or glucose.

The assay was set up by adding 0.5 ml of M9 without glycerol to all samples of dried CPS and transferring 0.5 ml of the glucose solutions to separate test tubes, then mixing 2.5 ml of anthrone reagent to all the samples. The tubes were capped with glass marbles and incubated at 100°C for 10 min, after which the samples were placed on ice to stop the reaction. The absorbance of the solutions was measured using a Spectronic20D spectrophotometer set at 625 nm, using M9 without glycerol as a blank.

RESULTS

Minimum Inhibitory Concentration. A minimum inhibitory concentration (MIC) assay was performed for each *E. coli* strain to determine the baseline for subsequent sub-lethal treatments of KAN. Generally, the mutant strains were found to have a lower MIC than the parental WG-1 strain (Table 2). Not surprisingly, growth of the AB301 double-mutant strain was inhibited at KAN concentrations 16x lower than that of the parental WG-1 strain. Interestingly, growth of the AT-2 *relA* mutant was inhibited at a concentration of KAN 2x lower than that of the 58-161 *spoT* mutant.

Growth of Pre-treated Strains. After sub-inhibitory KAN pre-treatment, growth in media containing inhibitory concentrations of KAN was measured to determine the extent of the induced resistance. The parental WG-1strain exhibited the most rapid increase in cell concentration throughout the time course of the experiment, increasing 5x in total cell concentration; on the opposite end of the spectrum, the double mutant AB301 exhibited no increase in cell concentration (Fig. 1). As for the 58-161 *spoT* mutant, it exhibited a 6x increase in total cell concentration while maintaining the second most rapid increase (Fig. 1). Finally, it should be noted that the AT-2 *relA* mutant exhibited a 3x increase in total cell concentration while increasing at a rate slower than the *spoT* mutant (Fig. 1)

Anthrone Carbohydrate Assay. CPS was quantified in order to compare the amount of induced CPS

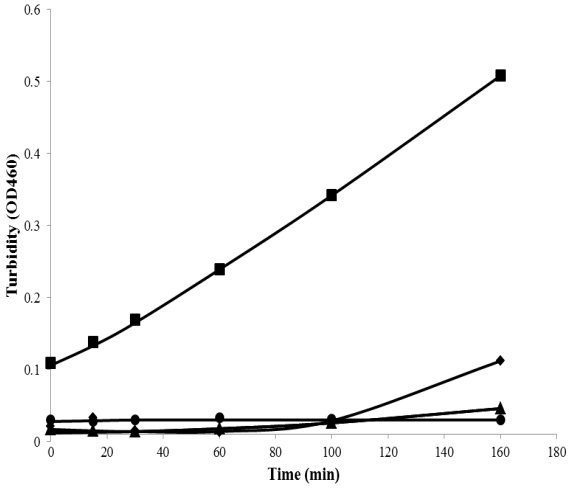

FIG. 1. Effect of KAN at 1.0x MIC on *E. coli* growth. WT (■), *relA* (▲), *spot* (♦) and *relA/spot* (●) strains were pre-treated at sub-lethal levels of kanamycin for an hour prior to the addition of kanamycin.

production across genotypes and across treatment conditions. For the pre-treated strains, the parental WG-1 exhibited a 25% greater concentration of isolated CPS than the double mutant AB301 (Fig. 2), suggesting that the combination of mutations in both *relA* and *spoT* had negligible effect on CPS production. However, the *relA* mutant AT-2 exhibited a 130% greater concentration of isolated CPS than the parental WG-1 (Fig. 2). No measurable concentrations of CPS were found for the *spoT* mutant 58-161 (Fig. 2).

As for the untreated strains, the total amount of CPS isolated was highest in the parental WG-1 (Fig. 3). This is followed by the double mutant AB301, which had a carbohydrate concentration 35% that of the parental WG-1. Next, the *relA* mutant AT-2 was found to have CPS at 40% that of the double mutant AB301 while finally, the *spoT* mutant 58-161 was found to have CPS at 40% that of the *relA* mutant AT- 2 (Fig. 3). As the cell concentrations of these strains were not measured prior to the quantitation of CPS, a standardization of CPS levels was not possible; however, the higher turbidities associated with the lack of KAN treatment suggest that the pre-treated strains produced more CPS on a per cell basis.

TABLE 4. Growth rates at t=160 min for *E. coli* strains as expressed by change in OD

	Strains			
	WT	*relA*	*spoT*	*relA/spoT*
Growth Rate (Δ/hour)	0.17	0.02	0.13	0.00

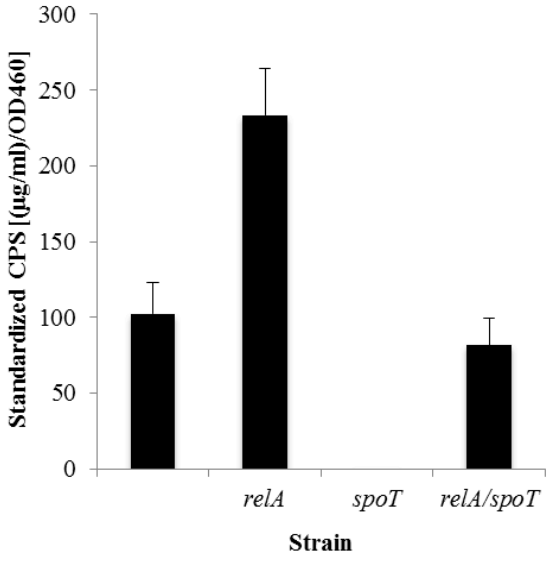

FIG. 2. Effect of pretreatment with sublethal levels of KAN on *E. coli* strains on CPS concentration. Carbohydrate concentrations as were standardized by measured cell concentration (OD_{460}) with error bars showing 95% confidence intervals.

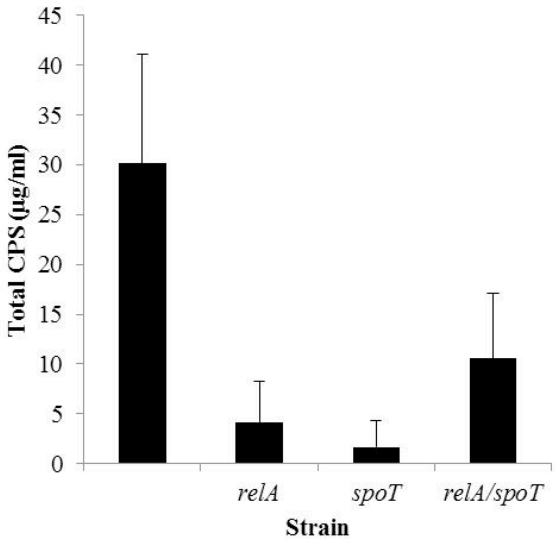

FIG. 3. DPS carbohydrate concentration of WG-1, AT-2, 58-161, and AB301 strains in the untreated condition. Error bars show 95% confidence intervals.

Capsular Polysaccharide Staining. In addition to the quantitative analysis of CPS production, a qualitative measure of CPS was performed via cell staining of different strains and treatment conditions. In general, cells that were pre-treated with sub-lethal levels of KAN exhibited visible induced CPS as shown by the presence of a white halo around the cells (Fig. 4). This white halo was not found around the cells of the control condition. Qualitatively comparing CPS induction across strains, this appeared to be much more prevalent for the WG-1 parental and AT-2 *relA* strains than it did for the AB301 double mutant strain and especially the 58-161 *spoT* strain.

DISCUSSION

The results of this experiment indicated a lack of induced CPS production in the 58-161 *spoT* mutant (Fig. 2). Due to the absence of ppGpp hydrolase in *spoT* mutants, an overall increase in ppGpp concentration results after pre-treatment with KAN (14). The excessive levels of ppGpp also results in the induction of the stringent response due to the over- expression of σ^S, allowing σ^S to dominate in competition with other sigma factors, such as sigma 70 (σ^{70}), which is predominant during exponential growth (8). σ^{70} is implicated in the regulation of the regulator of capsule synthesis (*rcs)* gene cluster, and in particular, the expression of RcsB (8). RcsB, in conjunction with RcsA, induces expression of the *cps* gene cluster and the production of CPS (12). Upon administration of KAN, the production of CPS was drastically limited as the induction of RelA activity and σ^S limited the activity of σ^{70}, which then limited the activation of *cps* genes. In addition to this competitive effect, σ^S has also been found to also exert a limiting regulatory effect on the *rcs* transcriptome, thus further attenuating the production of CPS (4, 7)

Conversely, CPS production was found to be highly upregulated in the AB301 *relA* mutant (Fig. 2); due to the lack of functional RelA, one would expect decreased amounts of ppGpp, even with the administration of KAN (19). As such, there should be decreased expression of σ^S, allowing for its transcription factor competitor σ^{70} to continue upregulation of CPS production via its effects on the *rcs* gene cluster. In the AB301 double knockout, it was found that a limited amount of CPS was still induced (Fig. 2) despite the absence of both functional *relA* and *spoT* gene products. It has been found that the administration of KAN also increases the activity of the environmental sensor RcsS (19). This factor, in conjunction with RcsB, has been implicated in the up-regulation of RprA, which in turn induces the expression of *rpoS* and σ^S (11,12). Thus, this would limit the production of CPS via the limitation of σ^{70} activity. Moreover, due to absence of both SpoT and RelA activity, it is expected that ppGpp levels would remain at a basal level, and as such, intra-cellular ATP levels would not be depleted due to the lack of

production or hydrolysis of ppGpp (19). This increase in intracellular ATP levels could increase the activity of two key ATP-driven regulatory proteases of the Lon and Clp family (17). Both Lon and Clp degrade RcsA, limiting its interaction with RcsB, and further preventing overabundant CPS production (17). With regards to the WG-1 parental strain, a high level of CPS was also induced following pre-treatment (Fig. 2), suggesting that a balance between SpoT and RelA activity resulted in an appropriate modulation of the stringent response via the regulation of ppGpp, allowing for regular production of CPS.

Untreated strains generally showed the same trend of CPS production (Fig. 3), with the difference in cell concentration accounting for the difference between the amount found in the AB301 double mutant and the AT-2 RelA mutant. However, there was still some CPS found in the untreated *spoT* mutant (Fig. 3) as compared to the lack of CPS found on the pre-treated SpoT mutant (Fig. 2). With the lack of environmental stress caused by the administration of KAN, it is possible that ppGpp remains largely at basal levels. Subsequently, σ^S should also be present at basal levels, limiting the competition for σ^{70} and resulting in homeostatic levels of CPS production. Thus, although carbohydrates were isolated from both the pre-treated and untreated conditions, it is probable that isolated CPS differs not only in overall abundance, but also in the composition and percentage of carbohydrates that actually confer resistance to aminoglycosides, which would drastically increase upon pre-treatment with KAN, while decreasing in the amount of carbohydrates which do not confer such resistance. This is supported with regards to the qualitative assessment of CPS production as the pre-treated strains exhibited presence of CPS compared to the relatively limited presence of CPS in the control conditions (Fig. 4). This discrepancy between our qualitative and quantitative evaluations of CPS is likely due to differences in these two methods of assessment. It is possible that the staining process is more specific for certain types of carbohydrates, which may be more efficacious in conferring resistance thus resulting in a discrepancy between our two methods of CPS assessment (10).

The up-regulation of CPS has also been implicated in conferring lessened sensitivity to aminoglycosides such as KAN (10, 13). Previous studies have found that CPS not only physically limits the entry of KAN into the cell, but it also sequesters KAN via an ionic interaction between the negative charges found on CPS and positive charges found on KAN (5, 13). A preliminary hypothesis would predict that the strains with the highest CPS production would have the largest decrease in antibiotic sensitivity upon pre-treatment with KAN. However, this was not found to be the case in our experiment. Our *spoT* mutant, which was found to exhibit the lowest amounts of CPS production, was found to have the second highest MIC following only the parental strain, along with the second highest growth rate (Table 3, Table 4). Thus, this suggests that there are other factors and pathways that play a critical role in the induction of aminoglycoside resistance other than the presence of CPS. As mentioned above, the lack of a functional ppGpp hydrolase in the *spoT* mutant

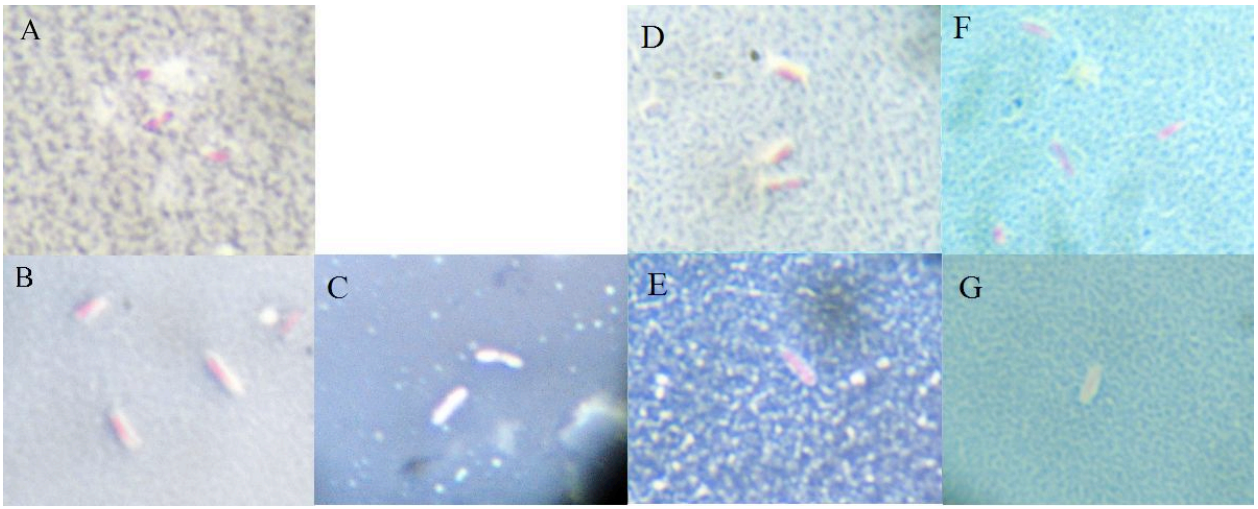

FIG. 4. Qualitative evaluation of the effect of sub-lethal treatment of kanamycin. Strains were either pre-treated with sub-lethal levels of kanamycin (bottom row) or no kanamycin for the control condition (top row). CPS was visualized for stratins WT (A and B), pre-treated *relA* (C), *spot* (D and E), and *relA/spot* (F and G).

results in increased cellular ppGpp concentration, which strongly upregulates the stringent response. Not only does the stringent response downregulate the production of CPS, but it also has been found to have a wide variety of other effects such as the regulation of central metabolism, amino acid biosynthesis and degradation, as well as nucleotide biosynthesis, which has the holistic effect of conserving cellular energy stores in order to ensure survival (3).

Moreover our results show that our *spoT* mutant exhibited both a higher baseline KAN MIC and a lower sensitivity to KAN following pre-treatment compared to our *relA* mutant (Table 3, Fig. 1). This suggests that the overall effects of ppGpp and stringent response confer greater protection against the activity of KAN than the induction and presence of CPS alone. This is possibly due to the lack of a stringent response in our *relA* knockout, which could lead to an increased sensitivity to KAN activity. Previous studies by Park *et al* where strains of *E. coli* were supplemented with additional supplementary isolated CPS have suggested that CPS confers a decreased sensitivity to KAN only up to concentrations of 7 µg/ml (13). The ionic interactions between CPS and the aminoglycoside that serve to sequester the antibiotic just outside the cell and confer resistance eventually lose efficacy with the presence of KAN at higher concentrations (13). Accounting for variability in the permeation threshold across strains, this could certainly explain the drastic drop-off in strain survival at the 2 MIC level of KAN. Our results also show that the *spoT* mutant displayed a lower MIC level than our parental control strain (Table 3, Fig. 1), which suggests that although the stringent response offers some protection against KAN, the presence of SpoT and other pathways leading from its activity, possibly including the induction of *cps,* results in higher KAN resistance. It is possible the induction of CPS is a side effect of resistance resulting from the activity of both RelA and SpoT, and its presence could be more of a correlative effect of resistance rather than a causative one. Our double knockout strain displayed virtually no decrease in sensitivity with pre-treatment and had the lowest MIC level among all four strains (Table 3, Fig. 1). This suggests that its deficiency in ppGpp production impeded the activation of the stringent response, resulting in a lack of protection against KAN as its baseline levels would be insufficient to activate this protective response. Also, CPS production was limited due to the previously mentioned alternate pathways, which may reflect a lack of induced resistance as an indicator of RelA and SpoT activity. It comes as no surprise that our double mutant strain exhibited negligible growth at a basal 1–MIC KAN level even after pre-treatment.

In short, our results indicate that a *spoT* mutant not only has a higher MIC of KAN than an isogenic *relA* mutant, but it also exhibited a faster growth rate when treated with higher doses of antibiotic following an initial sub-lethal pre-treatment. As expected, the highest MIC level and growth rate were displayed by the parental strain, while the lowest MIC level and growth rate were displayed by our double knockout strain. With regards to CPS quantification, it was found that our *relA* mutant displayed elevated levels of induced CPS after pre-treatment, while our *spoT* mutant displayed a lack of induced CPS production. As such, these results suggest that between these two regulators of ppGpp, RelA plays a larger role in conferring a decreased antibiotic sensitivity and facilitation of growth when compared to SpoT. However, the importance of CPS in conferring decreased sensitivity to antibiotics in mutant strains is limited and requires further characterization, as our results show that even with the lack of induced CPS found in our *spoT* mutant, this strain persisted and grew at a much higher rate than that of our *relA* mutant, which displayed the highest levels of induced CPS.

FUTURE EXPERIMENTS:

The discrepancies between our qualitative and quantitative analyses of CPS production by the different mutant strains of *E. coli* suggest that further analysis of the specific composition of CPS should be performed. Besides assessing total carbohydrate concentrations by the anthrone carbohydrate assay, more specific techniques to assay for particular types of carbohydrates could be performed. It is expected that the untreated conditions would produce different types of carbohydrates as compared to the pre-treated strains as a means of limiting aminoglycoside entry into the cell. Additionally, in performing the growth assay, it would be interesting to compare the growth curves not only after the pre-treatment phase, but also before and during the initial exposure to KAN during pre-treatment in order to further elucidate how the induction of capsular production and how the presence of sublethal levels of KAN affect growth across strains and treatment conditions to further confirm the effects of master transcription factors in the cell such as σ^{70} and σ^{S}. Untreated strains should exhibit growth consistent with standard exponential growth due to the absence of KAN, while pre-treated strains would exhibit growth limited in different orders of magnitude as a result of

these transcription factors produced in response to KAN.

To test the idea that CPS arises as a correlative rather than causative effect of antibiotic resistance, experiments could be performed where *relA* and *spoT* mutants would be supplemented with CPS isolated from isogenic wild-type strains pre-treated with KAN. Resistance to different concentrations of KAN would be measured using growth curves for these mutant strains supplemented with CPS. If the presence of CPS is simply correlated to antibiotic resistance, then the addition of CPS should confer only limited resistance; if CPS has more of a causative effect to resistance, then the addition of CPS should confer a significant increase in resistance.

Finally, mutations in select alternate genes implicated in CPS production such as the Lon or Clp protease family, which are also regulators of *rcs* and subsequently CPS production, would limit alternate activation of the *rcs* pathway and could help further pin down the specific roles of RelA and SpoT in their regulation of *cps* and the stringent response as compared to alternate means of regulation as suggested for our double mutant strain. It is expected that these protease mutants would have higher levels of induced CPS for the wildtype and the *relA* strain while the double *relA/spoT* mutant would have lower levels of induced CPS due to the lack of alternate *cps* induction.

ACKNOWLEDGEMENTS

We are most grateful of Dr. William Ramey for his invaluable advice and insight. We thank Laurence Madera for his guidance and suggestions. Also, we thank the media room for their technical expertise. This work was supported by the Department of Microbiology and Immunology at UBC.

REFERENCES

1. **Cheng, K., L. Ren, and K. Tsai.** 2010. Inhibitory concentrations of kanamycin in the presence of ppGpp synthase RelA confer protection against subsequent lethal antibiotic assaults in *E. coli* CP78. J. Exp. Microbiol. Immunol. **14:**51-56.
2. **Chiang, S., J. Koropatnick, J. Lam, and J. Tiu.** 2010**.** Salicylate treatment of *Escherichia coli* B23 with capsular polysaccharide induced by sub-lethal levels of kanamycin does not sensitize the Cells to T7 bacteriophage adsorption. J. Exp. Microbiol. Immuno. **14:** 92-97.
3. **Fung, D. K. C., E. W. C. Chan, M. L. Chin, and R. C. Y. Chan.** 2010. Delineation of a bacterial starvation stress response network which can mediate antibiotic tolerance development. Antimicrob. Agents Chemother. **54:**1082-1093.
4. **Gottesman, S., and V. Stout.** 1991. Regulation of capsular polysaccharide synthesis in *Escherichia coli* K12. Mol. Microbiol. **5:**1599-1606.
5. **Grant, W. D., I. W. Sutherland, and J. F. Wilkinson.** 1970. Control of colonic acid synthesis. J. Bacteriol. **103**: 89-96.
6. **Hengge-Aronis, R.** 2002**.** Signal transduction and regulatory mechanisms involved in control of the sigma S (RpoS) subunit of RNA polymerase. Microbiol. Mol. Biol. Rev. **66**: 373-395.
7. **Huang, Y., L. Ferrières, and D. J. Clarke.** 2006. The role of the Rcs phosphorelay in *Enterobacteriaceae*. Res. Microbiol. **157:**206-212.
8. **Jishage, M., A. Iwata, S. Ueda, and A. Ishihama.** 1996. Regulation of RNA polymerase sigma subunit synthesis in *Escherichia coli*: Intracellular levels of four species of sigma subunit under various growth conditions. J. Bacteriol. **178**:5447-5451.
9. **Kohanski, M. A., D. J. Dwyer, B. Hayete, C. A. Lawrence, and J. J. Collins.** 2007. A common mechanism of cellular death induced by bactericidal antibiotics. Cell **130:**797-810.
10. **Lu, E., T. Trinh, T. Tsang, and J. Yeung.** 2008**.** Effect of growth in sublethal levels of kanamycin and streptomycin on capsular polysaccharide production and antibiotic resistance in *Escherichia coli* B23. J. Exp. Microbiol. Immunol. **12**:21-36.
11. **Majdalani, N., D. Hernandez, and S. Gottesman.** 2002. Regulation and mode of action of the second small RNA activator of RpoS translation, RprA. **46:** 813-826.
12. **Majadalani, N. and S. Gottesman.** 2005. The Rcs phosphorelay: A complex signal transduction system**.** Annu. Rev. Microbiol. **59:** 379-405.
13. **Park, M., R. Sabet, and Y. Park.** 2010**.** The effect of isolated capsular polysaccharides on kanamycin sensitivity of *Escherichia coli* B23. J. Exp. Microbiol. Immunolol. **14:** 40-47.
14. **Sarubbi, E., K. E. Rudd, H. Xiao, K. Ikehara, M. Kalman, and M. Cashel.** 1989**.** Characterization of the *spoT* gene of Escherichia coli. J. Biol. Chem. **264**: 15074-15082.
15. **Srivatsan, A., and J.D. Wang.** 2008. Control of bacterial transcription, translation and replication by (p)ppGpp. Curr. Opin. Microbiol. **11:**100-105.
16. **Traxler, M. F., S. M. Summers, H. Nguyen, V. M. Zacharia, G. A. Hightower, J. T. Smith, and T. Conway.** 2008. The global, ppGpp-mediated stringent response to amino acid starvation in *Escherichia coli*. **68:** 1128-1148.
17. **Trisler, P., and S. Gottesman.** 1984**.** Lon transcriptional regulation of genes necessary for capsular polysaccharide synthesis in *Escherichia coli* K-12. J. Bacteriol. **160**: 184-191.
18. **Vinella, D., C. Albrecht, M. Cashel, and R. D'Ari.** 2005. Iron limitation induces SpoT-dependent accumulation of ppGpp in *Escherichia coli*. **56:** 958-970.
19. **Wendrich, T. M., G. Blaha, D .N. Wilson, M. A. Marahiel., and K. H. Nierhaus.** 2002**.** Dissection of the mechanism for the stringent factor RelA. Mol. Cell. **10:** 779-788.
20. **Whitfield, C.** 2006. Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*. Annu. Rev. Biochem.**75:**39-68.
21. **Wu, J., and J. Xie**. 2009. Magic spot: (p)ppGpp. J. Cell. Physiol. **220:**297-302.