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Inhibitory Concentrations of Kanamycin in the Presence of ppGpp Synthase RelA Confer Protection Against Subsequent Lethal Antibiotic Assaults in *E. coli* CP78

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Treatment of bacteria with antibiotics is known to cause persistence. In this persistence state, the cells that survived ceased to grow and became resistant to antibiotics. The mechanism of such protection however, was never elucidated. Utilizing isogenic *E. coli* strains CP78 and CP79 pretreated with 9 µg/mL of kanamyin for 1 hour in M9 minimal media, we invistigated whether or not ppGpp synthase I (RelA) in *E. coli* CP78 pretreated with an inhibitory amount of kanamycin are responsible in eliciting cross-protection against subsequent lethal antibiotic insults the different antibiotics Tetracyclin and Ampicilin. RelA was found to be important in the viability of the cells during the kanamycin pretreatment and the subsequent lethal antibiotic treatments: CP78 and CP79 strains both started with similar turbidity and cell counts prior to the kanamycin treatment; after 1 hour treatment the CP78 strain was found to have 28% more cells that survived the pretreatment compared to the CP79 strain. After the secondary lethal antibiotic treatments, the RelA carrying strain CP78 was found to be at least 3 times or more resistant to tetracycline or ampicillin killing compared to its isogenic strain, CP79 strain, which carries a relA mutation. We conclude that the presence of RelA could provide short-term (1.5 hours) protection against antibiotics such as kanamycin, and that relA in conjunction with a pretreatment of inhibitory level of kanamycin was able to provide short-term protection (2.5 hours) against subsequent lethal levels of antibiotic insults from different classes of antibiotics.

Previous report by Chen *et al.* (8) reported that treatment of *E. coli B23* with a sub-lethal amount (7 µg/mL) of kanamycin could elicit both short (15 min+) and long term (24 hr+) protection against lethal amounts of antibiotics of different classes. Up-regulated capsular polysaccharide production and alteration of composition of outer-membrane porin were the proposed mechanism for such resistance, the real mechanism however, was not elucidated.

The alteration of the composition of outer-membrane porins and the up-regulation of capsular polysaccharide production can both be modulated by RelA induced stringent response (9,10). RelA is a ribosome associated enzyme responsible for producing the alarmone, guanosine tetraphosphate, or ppGpp, which is central for the induction of stringent response (Ref). When amino acid is scarce in the environment, ribosomes could become stalled if a certain amino-acyl tRNA species is low in concentration. During this stalling, if a uncharged tRNA wanders into the A site, this uncharged tRNA-ribosome complex could cause RelA to bind to the complex and become activated. Activated RelA produces ppGpp, which in-turn will initiate stringent response. Cells undergoing stringent response would cease to grow, rendering antibiotics which target DNA replication, protein synthesis and peptidoglycan synthesis ineffective. In addition, up regulated capsular polysaccharide synthesis coupled with decreased outer-membrane permeability provide additional protection against antibiotics.

Based on what we know already about RelA and the mechanism of kanamycin, we propose that RelA plays a key role in protecting the cells against antibiotic insults: Presumably, in the experiments performed by *Chen et al*, the sub inhibitory concentration of kanamycin caused a population of the bacteria ribosome to stall, and those stalled ribosome in combination with uncharged tRNA activated RelA. The resulting stringent response then protected the cells against subsequent lethal antibiotic insult.

To test this hypothesis, we pretreated treated relA carrying Strain *E. coli* CP78 and its isogenic relA negative mutant strain CP79 with an inhibitory concentration of kanamycin (9 µg/mL) for one hour to determine the protective effects of *rel*A. We then split the pretreated cultures into subcultures and treated separately with 1.0 MIC, 1.5 MIC, and 2.0 MIC of either tetracycline or ampicillin for an additional hour.

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Should RelA be protective against inhibitory kanamycin treatments two outcomes were expected.: Firstly, the survival rates measured by both OD660 and plate counts of CP78 after the one hour treatment should be significantly higher than that of CP79, conversely, if RelA offered no protection against kanamycin survival rates of both strains should then be similar. Secondly, the survival rates obtained after secondary lethal antibiotic treatments should be exaggerated should RelA be protective against kanamycin pretreatment: RelA carrying CP78 would have more viable cells compared to RelA- CP79 at the beginning of the secondary antibiotic treatments. Conversely, if RelA offered no protection

If an inhibitory concentration kanamycin could activate RelA and thus the stringent response, survival rates of CP78 after lethal antibiotic treatments should be significantly higher than that of CP79, conversely if RelA offered no protection, the survival rates of both strains were expected to be similar.

MATERIAL AND METHODS

Bacterial strains. Isogenic strains of *E. coli* K12 CP78 (F-, *thr-1*, *leuB6*(Am), *fhuA2*, *glnV44*(AS)?, *gal-3*, *his-65*, *malT1*(λ^R), *xyl-7*, *mtlA2*, *argH46*, *thi-1)* and CP79 (F-, *thr-1*, *leuB6*(Am), *fhuA2*, *glnV44*(AS)?, *gal-3*, *his-65*, *relA2*, *malT1*(λ^R), *xyl-7*, *mtlA2*, *argH46*, *thi-1)* were obtained from the MICB 421 culture collection from the Microbiology and Immunology Department at the University of British Columbia, and grown at 37 ^oC using vitamin and amino acids supplemented M9 media.

Preperation of LB plates. LB agar plates were prepared by combining 5 g of tryptone (BD Bacto), 2.5 g of yeast extract (BD Bacto), 1 g of glucose (Sigma), 5 g of sodium chloride (Fisher Scientifics) and 7.5 g of Select Agar (Invitrogen) with 500 ml of distilled water in an 1L Erlenmeyer flask and adjusted to pH 7.2 by adding 1N NaOH. The mixture was then autoclaved and poured.

Preparation of sterile amino acids and vitamin mixture. 0.31 g of thiamine (Sigma T-4625) 0.21 g of arginine (Difco 0583-12), 0.22 g of histidine (Pierce 20065), and 0.23 g of leucine (Sigma L-8000) were dissolved in 62 ml of distilled water and autoclaved.

Preparation of M9 minimum salt media with amino acids and vitamin supplement. Solution A is made by dissolving 0.25 g of NaCl (Fisher Scientifics), 3.5 g of Na_2HPO_4 (Sigma), 1.5 g of KH_2PO_4 (Fisher Scientifics), 0.5 g of NH_4Cl in 400ml of distilled water.

Solution B is made by dissolving 0.1 g of MgSO4.7H2O (Fisher Scientifics) and 1 g of glycerol(MICB 421 Preperations) in 20 ml of distilled water. Enough sterile amino acids/vitamin mixture was added so that the final concentration in 500 ml was 40 mg in arginine, histidine, and leucine. Enough thiamine was added so that the final concentration in 500 ml was 5 mg/ml.

Solution A and Solution B were autoclaved separately, cooled then combined and made up aseptically to 500 ml with sterile distilled water.

Preparation of sterile antibiotic solutions. 0.15625 g of tetracycline (Sigma T-3383) was dissolved in 16 ml of distilled water to make the final concentration of 15.625 mg/ml. 0.2 g of kanamycin (Sigma K-4000) was dissolved in 7 ml of distilled water to make the final concentration of 28.57 mg/ml. 0.31g of ampicillin (Sigma A-9518) was dissolved in 11 ml of distilled water to make the final concentration of 27.27 mg/ml. The antibiotic solutions were then filter sterilized using sterile milipore 0.45um filter cups into sterilized test tubes. Aluminum foils was wrapped around the tube containing tetracycline to prevent photo-degradation. The antibiotic solutions were then stored in -20 ^oC for further use.

MIC Assay. Overnight cultures of E. coli CP78/79 were combined with fresh M9 media supplemented with amino acids and vitamins to obtain an OD460 reading of 0.01. 100 µl of the inoculums were then transferred into each well of Sarstedt sterile 96 well plates. The liquid levels of each of the plates were then checked to ensure that each well received 100 µl of the inoculums. 50 µl of sterile M9 media with amino acids and vitamins were then added into each of the wells to make the total volume 150 µl. Enough antibiotic solutions was added into sterile 10 ml of M9 media with amino acids and vitamin so that the final concentration when 50 µl of it is added into the 150 µl innoculum the resulting concentration is 512 µg/ml. The 10 ml aliquot of kanamycin containing M9 media was then poured into a sterile multi-channel pipettor reservoir. A multi-channel pipettor outfitted with 3 tips were used to pipet 50 µL of the kanamycin containing M9 media into wells A1, B1, and C1 of both CP78 and CP79 plate. The wells were mixed by pipetting then 50 µl of the innoculum was transferred into wells A2, B2 and C2 and mixed. The process was repeated until all the wells from the first three roles were inoculated with kanamycin. M9 media containing tetracycline was then prepared following the procedure illustrated above, and 50 µl of it was transferred to wells D1, E1, F1 of both plates. Following the procedure illustrated above, tetracycline was serially diluted through rows D, E, and F. M9 media containing ampicillin was used to inoculate rows G and H following the procedure illustrated above. The plates were then incubated in 37 ^oC incubator for 24 hours. The minimum inhibitory concentration (MIC) of the respective antibiotics was then determined by looking for the wells with the lowest concentration of a particular antibiotic that had no bacterial growth.

Radioactivity Assay. The radioactivity incorporation assay was performed alongside the turbidity reading using 6 ml of the cell cultures and 0.50 µCi ^{14}C-uracil (Sigma). Turbidity readings measured using a Spectronic 20 spectrophotometer. 50 µl of sample was taken several times and transferred to the filter disks (Whatman 1003-323) in replicates. The incorporated radioactivity was precipitated in the disks with 5% trichloroacetic acid. The soluble label was washed away with two volumes of clean trichloroacetic acid. The disks were then dried and counted.

Determination of Secondary Antibiotic Cross Protection. The two strains were subjected to inhibitory amount of kanamycin which was 9 µg/ml for 60 min 37 ^oC. The turbidity of the treated cells were then measured and the cells were plated onto LB agar plates. The treated cells were then split into separate subcultures and treated separately with 0.5 x, 1.5 x or 2.5 x MIC of either tetracycline or ampicillin for 60 mins at 37 ^oC. Turbidity readings of the treated cells were taken at the end of the incubation period and the treated cells were then plated onto LB agar plates. A shaking water bath set at 200 rpm and 37 ^oC was used during the treatment of the cultures, the LB plates were incubated in an incubator set at 37 ^oC.

RESULTS

Growth Rate. The growth curve determined for *E.coli* CP78 and CP79 showed exponential increase in cell concentration over the 90 minute incubation time. According to Figure 1, the general growth trends for the two strains of *E.coli* were parallel which indicates that they are the same in terms of growth pattern. The doubling time for both E.coli CP78 and CP79 is 66 min. The turbidity readings taken for E.coli CP78 at 35 min and 81 min time point were excluded from the growth curve which can be explained by the fluctuation of the Spec. 20 machine.

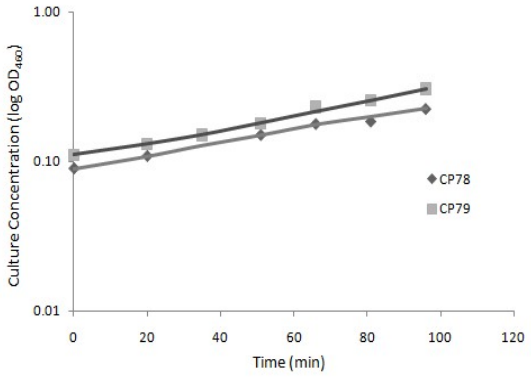

FIG. 1. The growth rate of *E.coli* CP78 and CP79 in modified M9 salt media was measured.#

Minimum Inhibitory Concentration. The minimum inhibitory concentration (MIC) of kanamycin(32 µg/ml) and ampicillin (16 µg/ml) were determined to the same in both CP78 and CP79 (Table. 1). The MIC of tetracycline (Table. 1), however, was determined to be 8 µg/ml in *E. coli* CP78 and 4 µg/ml in CP79 (Table. 1). The result was as expected since *E. coli* CP79 which was *relA*$^-$, therefore was expected to be less tolerant than *E. coli* CP78 due to the lack of stringent response (23).

The survival rate of both strain CP78 and CP79 after one hour of 9 µg/ml kanamycin treatment was similar: more than 99% of the cells were killed, indicating this concentration was inhibitory.

Radioactivity Incorporation Assay. The effect of kanamycin on cell growth was identical for both *E. coli* CP78 and CP79 controls (Figure. 2). The general trends for the controls samples showed parallel increase over time. The test samples did not show an increase in turbidity as expected. The *E .coli* CP78 strain treated with kanamycin had a linear trend line indicating a cessation in cell growth. The *E. coli* CP79 strain treated with kanamycin, showed a slight decrease in turbidity over time which might mean that some cells were lysed during the treatment due to a lack of the *relA* gene that had some unknown protective functions against kanamycin.

In figure 3, the radioactivity incorporation normalized to OD_{460} was used to measure RNA synthesis in the two *E. coli* strains treated with or without kanamycin treatment. The graph clearly showed an increase in radioactivity incorporation in both of the control samples which contained no

TABLE 1. Minimum inhibitory concentration determined for *E.coli* CP78 and *E.coli* CP79.

	Minimum Inhibitory Concentration (µg/ml)	
	E.coli CP78	*E.coli* CP79
Kanamycin	32	32
Tetracycline	8	4
Ampicillin	16	16

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kanamycin. The two trend lines are nearly parallel with the *E. coli* CP78 control sample showing a linear trend line between 15 min and 30 min. The variation was contained in the 16% uncertainty for the radioactivity. In general, *E.coli* CP78 treated with kanamycin showed linear trend after 45 min. The *E. coli* CP79 strain treated with kanamycin showed a slow increase after 45 min which was inconsistent with the expectation. As a relaxed strain, CP79 should continue incorporating uracil after the addition of kanamycin, and the rate of incorporation should be similar compared to the CP79 strain without kanamycin

Antibiotic susceptibility. *E. coli* CP78 and CP79 were incubated with 9 µg/ml kanamycin for 60 min and then treated with either ampicillin or tetracycline for 90 min. The cell survival for *E. coli* CP78 (0.16 %) was 25% better than that of CP79 (0.12%) (Table 2). This is consistent with the expectation that *E. coli* CP78 would be more tolerant to the antibiotics than CP79 due to the *relA* gene.

DISCUSSION

The MIC determined using sterile polystyrene 96 well plates may have been wrong because polystyrene might form van der waal interactions with antibiotics and prevent the antibiotic from being dissolved into solution, resulting in a higher than actual MIC readout. This problem was not discovered until all the experiments were carried out and may have affected the accuracy of the reported MIC, however, the protective effect of *rel*A was clearly demonstrated despite the possibility of higher concentrations of antibiotics being used. The use of polycarbonate plates instead of polystyrene plates will remedy the problem for future experiments

TABLE 2. Cell survival measured for *E.coli* CP78 and CP79 treated with 9 µg/ml of kanamycin for 60 minutes.

	Cell concentration before kanamycin treatment (cfu/ml OD)	Cell concentration after kanamycin treatment (cfu/ml OD)	cell survival (%)
E.coli CP78	4.3×10^8	6.9×10^5	0.16
E.coli CP79	4.1×10^8	5.0×10^5	0.12

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TABLE 3. Cell survival measured for *E.coli* CP78 and CP79 treated with 9 µg/ml amount of kanamycin for 60 minutes and then subjected to 1.0, 1.5, and 2.0 MIC of ampicillin for 90 minutes.

	Colony Forming Unit (cfu/ul)					
Incubation time (minute)	*E.coli* CP78 (10^5 cfu/µl)			*E.coli* CP79 (10^3 cfu/µl)		
	1.0 MIC	1.5 MIC	2.0 MIC	1.0 MIC	1.5 MIC	2.0 MIC
0	5.0 +	5.0 +	5.0 +	2.6	2.1	2.1
30	5.0 +	5.0 +	5.0 +	2.2	2.4	2.0
60	5.0 +	5.0 +	5.0 +	1.5	1.6	1.2

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The protective effect of RelA in antibiotic resistance was demonstrated by the plating assays. The CP78 strain incubated with an inhibitory concentration of kanamycin for one hour was shown to survive 25% better compared to CP79 using OD normalized plate count data (Table.2). The difference in cell viability was further exaggerated by the subsequent one hour treatments of lethal dosages of tetracycline and ampicillin. All the CP78 plates at 10^{-1} plating factor after the secondary treatment with either tetracycline or ampicillin had bacterial lawn and was estimated to have 5.0 x 10^5 cfu/µl or more cells on them, in contrast CP79 after one hour tetracycline treatment had 1.2-1.5 x 10^3 cfu/ul on them depending on the MIC used, and CP79 after one hour of ampicillin treatment had 2.6-2.9 x 10^3 cfu/ul on them (Table. 3). The overall population of viable cells declined over the course of the treatment of one hour as expected, but there were no observable dosage dependent effects on the different concentrations of different antibiotic used: the range of 1.0 MIC to 2.0 MIC might have been too narrow to properly observe the antibiotic sensitivity in a dosage dependent manner.

Despite strong indications from the results of plating assays that the cross protection against antibiotics was due to *rel*A being activated by the treatment of kanamycin, evidence of *rel*A activation could not be seen clearly in the radioactivity incorporation graphs. The CP78 strain treated with kanamycin was expected to show impaired radioactive uracil incorporation, yet at the beginning of the incubation period in Fig. 3 and Fig. 4, the CP78 strain was seen taking up radioactive uracil at a steady and more rapid rate than the CP79 strain. This surge of uracil uptake ended in about 10 minutes after it was first seen and then the rate tapered off and the level of radioactivity stayed constant indicating that the incorporation of radioactive uracil was stopped. Unlike the CP79 strain, which incorporated slightly more radioactivity compared to the CP78 strain at the 20 minutes mark, the CP78 strain did not start taking up radioactive uracil until about 20 minutes. This lag in radioactivity uptake could be explained by the activation of RelA and other stress response proteins at the beginning of the treatment resulting halting of rRNA synthesis (20). The up-regulation however, was not by very much according to Traxler *et al*, and may help to explain the delay uptake of uracil at the 20 minutes mark: the up-regulation of the central metabolic pathway increased the strength of proton-motive force, which in-turn drove the uptake or uracil. The mechanism behind the increase radioactive uracil uptake by strain CP78 remained elusive and will have to be elucidated by further experiments. Current experimental data suggested that *rel*A was at least partly responsible for the phenomenon observed.

TABLE 4. Cell survival measured for *E.coli* CP78 and CP79 treated with sub-lethal amount of kanamycin for 60 minutes and then subjected to 1.0, 1.5, and 2.0 MIC of tetracycline for 90 minutes.

	Colony Forming Unit (cfu/ul)					
Incubation Time (minute)	*E.coli* CP78 (10^5 cfu/µl)			*E.coli* CP79 (10^3 cfu/µl)		
	1.0 MIC	1.5 MIC	2.0 MIC	1.0 MIC	1.5 MIC	2.0 MIC
0	5.0 +	5.0 +	5.0 +	3.9	3.9	3.3
30	5.0 +	5.0 +	5.0 +	3.1	3.2	3.1
60	5.0 +	5.0 +	5.0 +	2.6	2.5	2.9

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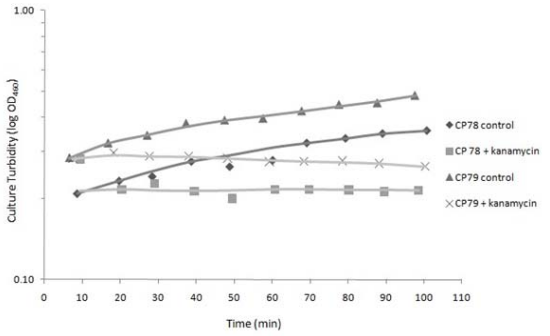

FIG. 2. Effect of kanamycin on th growth of *E.coli* CP78 and CP79. The test samples contain 9 µg/ml of kanamycin whereas the control samples contain no kanamycin.#

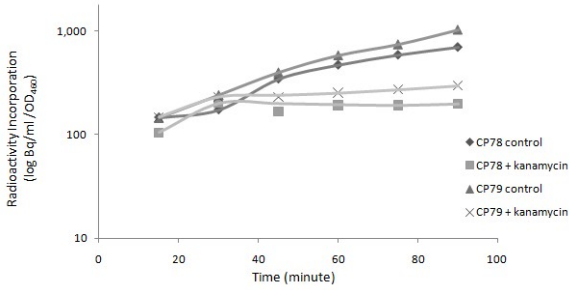

FIG. 3. Effect of kanamycin in ^{14}C-uracil incorporation by *E. coli* CP78 and CP79 .#

In contrast to the delayed uptake of radioactive uracil, the kanamycin treated CP79 strain had already incorporated higher radioactive uracil by 20 minutes after kanamycin treatment. This could partly be attributed to its inability to initiate stringent response (absence of radioactive uracil incorporation rate decrease seen in treated CP78, Figure 3 and 4). However, despite the non-functional relA gene, the rate of radioactive uracil incorporation was still much lower compared to the untreated control which indicated that there were other mechanisms at work repressing the uptake of radioactive uracil. It was demonstrated by Kohanski *et al.* that kanamycin, quinolones, and β-lactames all could kill by a common iron catalyzed mechanism (17), and SpoT was found to be able to respond to iron related stress in a separate report by Vinella *et al.* (21), it is therefore possible that treatment of kanamycin could induce the activation of SpoT through an iron stress dependent mechanism and that the ppGpp produced could exert their influence in transcription initiation through other ppGpp dependent mechanisms such as *dks*A. However, since DksA functions as a ppGpp driven modulator for RNA polymerase, which affects the transcription of the rRNA/tRNA genes (12, 15), its effect be it in combination with RelA or stand alone on the incorporation of nucleotides remained unclear: high cellular concentration of un-polyermized uracil could also exert end-point inhibition on the further uptake of uracil. Inhibition of protein synthesis by kanamycin coupled with existing uracil transporter degradation could also prevent the uptake of radioactive uracil. More experiments would likely be required to elucidate the true mechanism behind the inhibition of radioactive uracil incoproration in *E. coli* CP79 after treatment with inhibitory amount of kanamycin.

In this report we conclude that RelA protects *E. coli* CP78 against an inhibitory concentration of kanamycin and subsequent lethal tetracycline/ampicillin assaults partly through the induction of stringent response. Although the stringent response could not be clearly seen possibly due to the interference from SpoT and DksA, the survival rate between CP78 and CP79 after treatment was evident enough that RelA played an important role in the survival of the bacteria. We can assume that the protection from RelA is rendered through a 'stringency like response' after kanamycin treatment as indicated by Han *et al.* however, more experiments using a more elaborate *E. coli* strain are needed to confirm this hypothesis.

FUTURE DIRECTIONS

In order to fully visualize the effect of *relA* induced by inhibitory amounts of kanamycin, a set of isogenic strains of *E. coli*, which both strains are lacking *spo*T and *dks*A while one strain contains a functional *relA* gene and the other lacks it, is required. The elimination of *spo*T will ensure that no guanosine tetraphosphate is produced other than from RelA, therefore eliminating any background level of stringent response caused by SpoT, enhancing the clarity of the outcome. The double knockout in *relA* and spoT would abolish the transcription of amino acid synthetic genes of 11 amino acids because their transcription requires certain amount of ppGpp (20).

The elimination of *dks*A will ensure that the incorporation of radioactive uracil is not disrupted by the presence of guanosine tetraphosphate should there be some other unknown proteins that can synthesize guanosine tetraphosphate.

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