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Sublethal Kanamycin Induced Cross Resistance to Functionally and Structurally Unrelated Antibiotics

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Previous studies have shown that pretreatment with sub-lethal kanamycin to *E. coli* B23 cells provided protection against subsequent exposure to lethal amounts of the same antibiotic. It has also been shown that exposure to sub-inhibitory levels of certain antibiotic can induce cross resistance by promoting the development of low-level resistance to other functional and structural unrelated antibiotics. In this study, the kanamycin protection experiment by Lu *et al.* was reproduced using Minimal Inhibitory Concentration (MIC) assays in microplates to assess antbiotic resistance. The potential for development of cross resistance against streptomycin, tetracycline and ampicilin elicited by sublethal kanamycin pretreatment was also investigated. Short term protection was assessed by monitoring the growth rate in the presence of antibiotic for 1.5 hours, whereas long term protection was determined by the shift in the MIC of each antibiotic after 24 hours incubation. Both short and long term protection against kanamycin were found to be induced in the sublethal kanamycin pretreated culture. It was also observed that kanamycin pretreated *E. coli* B23 cells developed short term antibiotic resistance against streptomycin, tetracycline and ampicillin, whereas long term resistance was only observed against kanamycin and streptomycin. Thus suggesting different mechanisms may be involved in the short and long term protection.

Despite over 70 years of clinical antibiotics use, bacteria continue to out-perform antibiotics by developing increasing levels of resistance. *In vitro* and *in vivo* studies suggest that inappropriately low antibiotic dosing may be contributing to the increasing rate of antibiotic resistance (17). Previously, Lu *e al* investigated the chance in susceptibility of pretreated *E.coli* B23 cells with sublethal amount of kanamycin or streptomycin to a subsequent exposure to lethal amount of the same antibiotics (11). They observed that the pretreated cells developed increased antibiotic resistance relative to untreated cells (11). An *in vivo* model by Fung-Tomc *et al* found that the exposure of methicillin-resistant *Staphylococcus aureus* to sub-inhibitory levels of ciprofloxacin promoted the development of low-level resistance to tetracycline, imipenem, fusidic acid, and gentamicin; pre-exposure of ciprofloxacin to a *P. aeruginsa* variant promoted decreased susceptibilities to imipenem, amikacin, and cefepime (5). Consistent results were also found in a similar study using *S. pneumonia* (8). All of these findings suggest that exposure to sublethal levels of certain antibiotics could also protect against other functional and structural unrelated antibiotics, an observation we refer to as cross resistance.

In this study, we set out to confirm their results using different methods. Furthermore, we assessed the development of transient (short term) cross-protection in *E. coli* B23 cells exposed to lethal amounts of streptomycin, tetracycline and ampicillin after pre-exposure to sublethal levels of kanamycin, and also assess whether this protection persists over a long period of time. Three classes of antibiotics were used in our experiment, which were aminoglycosides, tetracyclines and β-lactams. Kanamycin and streptomycin belong to the class of antibiotics aminoglycosides, which target the 30S ribosomal subunit. They inhibit the translocation of the peptidyl-tRNA from the A-site to the P-site, cause misreading of mRNA and leave the bacterium unable to synthesize proteins vital for its growth (10, 11, 14). Tetracycline, another class of antibiotic, also targets on 30S ribosomal subunit in the mRNA translation complex. However it blocks protein synthesis by inhibiting the binding of aminoacyl-tRNA to the mRNA-ribosome complex (18). The β-lactam antibiotic ampicillin acts as a competitive inhibitor of the enzyme transpeptidase which is needed by bacteria to make their cell walls (9). Ampicillin inhibits the third and final stage of bacterial cell wall synthesis, which ultimately leads to cell lysis.

MATERIALS AND METHODS

Bacterial strains and culture methods. *Escherichia coli* B23 was obtained from the MICB 421 culture collection in the Microbiology

and Immunology Department of the University of British Columbia. An overnight culture was prepared by inoculating 5 ml of Luria broth (LB) (Tryptone 10.00 g; yeast extract 5.00 g; NaCl 10.00 g; distilled water, 1000 ml [pH 7.0]) with a loopful of *E. coli* B23 in a test tube. The inoculated culture was placed overnight on shaking platform at 170 rpm in 37°C incubator. 1 ml of the overnight culture was then inoculated into two flasks with 19 ml LB. The newly inoculated cultures were grown for 4 hours to an OD_{595} of 1.0 by incubating them on shaking platform at 170 rpm in 37°C incubator.

Antibiotic stock solutions. Kanamycin monosulfate (Sigma #K4000), streptomycin sulphate (Sigma #S6501-50G) and ampicillin sodium (Sigma #A-9518) were prepared by dissolving each antibiotic in distilled water to a final concentration of 20 mg/ml. Tetracycline hydrochloride (Sigma #T-3383) was prepared by dissolving in 70% ethanol to a final concentration of 25 mg/ml. All solutions were then filter sterilized using a Millipore 0.22 μm nitrocellulose membrane (GSWP 01300). Sterilized antibiotic solutions were stored at -20°C.

Sub-inhibitory antibiotic pretreatment. Kanamycin stock solution was added to one newly inoculated 20 ml culture to create a 4 μg/ml kanamycin pretreatment condition. The other 20 ml culture served as a control condition. Both cultures were incubated for an additional 1 hour on a shaking platform at 170 rpm in a 37°C incubator. By using LB as a blank, turbidity was measured after the 1 hour incubation with a Spectronic 20D spectrophotometer set at a wavelength of 595 nm and the turbidity readings for both cultures were equalized. The concentration of viable cells in both cultures was then estimated by spread plating in duplicate at final plated dilutions of 10^{-7}, 5×10^{-8} and 10^{-8} respectively. Before the start of the minimal inhibitory concentration (MIC) assays, to the 20 ml control culture, kanamycin stock solution was added to create the same final concentration (4 μg/ml) as in the pretreated culture to equalize the amount of kanamycin present in both cultures for subsequent MIC assays.

Preparation of antibiotic working solutions. To 1.5 ml eppendorf tubes, sufficient volumes of kanamycin stock solution were added to produce 1 ml of antibiotic working solutions, each with final concentrations of 2, 4, 8, 12, 16, 19.2, 22.4, 25.6, 28.8 and 32 μg/ml. Luria broth was added to bring the final volume to 1 ml. The same procedure was used to prepare streptomycin and tetracycline working solutions. Ampicillin working solutions was prepared by the same methods with final concentrations of 0.5, 1, 2, 3, 4, 4.8, 5.6, 6.4, 7.2 and 8 μg/ml.

Transient antibiotic resistance assay. To two 96 well flat bottom plates (Sarstedt # 82.1581.001), 100 μl of each prepared antibiotic working solutions were added in duplicate to each plate. Bacterial cultures were diluted to an OD_{595} of ~ 0.4, and 100 μl of pretreated culture was added to one plate, and control culture to the other to obtain a final starting OD_{595} of 0.20. Controls were set up by adding 100 μl of each culture to 100 μl of LB that contained no antibiotics. Samples were then incubated in 37°C incubator. The growth of culture was then monitored every 20 minutes by measuring the turbidity by using Bio-RAD Model 3550 Microplate reader in 37°C incubator for 1.5 hours, with Luria broth as a blank.

Determination of minimal inhibitory concentrations. The same procedure from transient antibiotic resistance assay was used to set up another two 96 well flat bottom plates with initial OD_{595} around 0.005. The samples were then incubated for 24 hours in the dark in 37°C incubator. Turbidity readings were obtained using a Bio-RAD Model 3550 Microplate reader the next day. The Minimal Inhibition Concentration of each antibiotic for *Escherichia coli* B23 cells was estimated by the lowest concentration at which absence of growth was observed.

Mathematical analysis of growth rate. The growth curve was plotted with turbidity readings obtained during the 1.5 hours monitoring at various concentrations for each antibiotic. The growth rate was estimated by taking the tangent to the growth curve at 20 minutes and 60 minutes respectively. The ratio of estimated growth rate of pretreated culture to that of untreated culture was then calculated.

RESULTS

Figure 1 showed that at 20 minutes after treatment, the growth rate measured in kanamycin pretreated samples were similar to these in untreated samples at various concentrations for all four antibiotics. The ratio of pretreated growth rate to untreated growth rate is constant around 1.0. However, in general, the growth rate of kanamycin pretreated sample is higher than that of untreated sample at 60 minutes after treatment. For kanamycin and ampicillin, the ratios stayed relatively constant at low antibiotic concentrations, and start to increase at high antibiotic concentrations. The same general trend in ratio was also observed for streptomycin and tetracycline, except the ratio reached a maximum and started to decrease. Among all the antibiotics, ampicillin treatment showed the highest ratios at all concentrations, with highest ratio being 12%, 16% and 44% higher than those observed in kanamycin, streptomycin and tetracycline respectively. In addition, the calculated ratios stayed relatively constant at various ampicillin concentrations compared to other antibiotic treatments.

A 50% up-shift in MIC of kanamycin and streptomycin was observed in kanamycin pretreated sample compared to untreated sample. However, the MIC of tetracycline and ampicillin stayed unchanged. The turbidity was equalized to around 1.1 for both cultures before spread plating. Countable results were obtained at the final plated dilution equal to 5×10^{-8}, with an average of 62 and 66 in pretreated and untreated cultures respectively. The spread plating results suggested similar concentrations of viable cells were present after 1 hour pretreatment incubation.

DISCUSSION

Both transient and long term protection against kanamycin induced by subinhibitory kanamycin pretreatment were observed in our experiment (FIG. 1) which agrees with the study by Lu *et al* (11). As seen in FIG. 1, the combined results for short term antibiotic treatments at different concentrations demonstrated that a sub-lethal concentration of kanamycin also induced cross resistance to lethal doses of streptomycin, tetracycline and ampicillin respectively. Long term resistance was also observed for streptomycin but not for tetracycline and ampicillin. These findings suggest that the mechanism of adaptation to kanamycin pretreatment might have caused a long term effect in *E. coli* B23's resistance to aminoglycosides but not to tetracycline and ampicillin.

There are a number of possible explanations for the observed transient protection against antibiotics after being treated with a sub-lethal level of kanamycin. Protection against antibiotics probably involves the

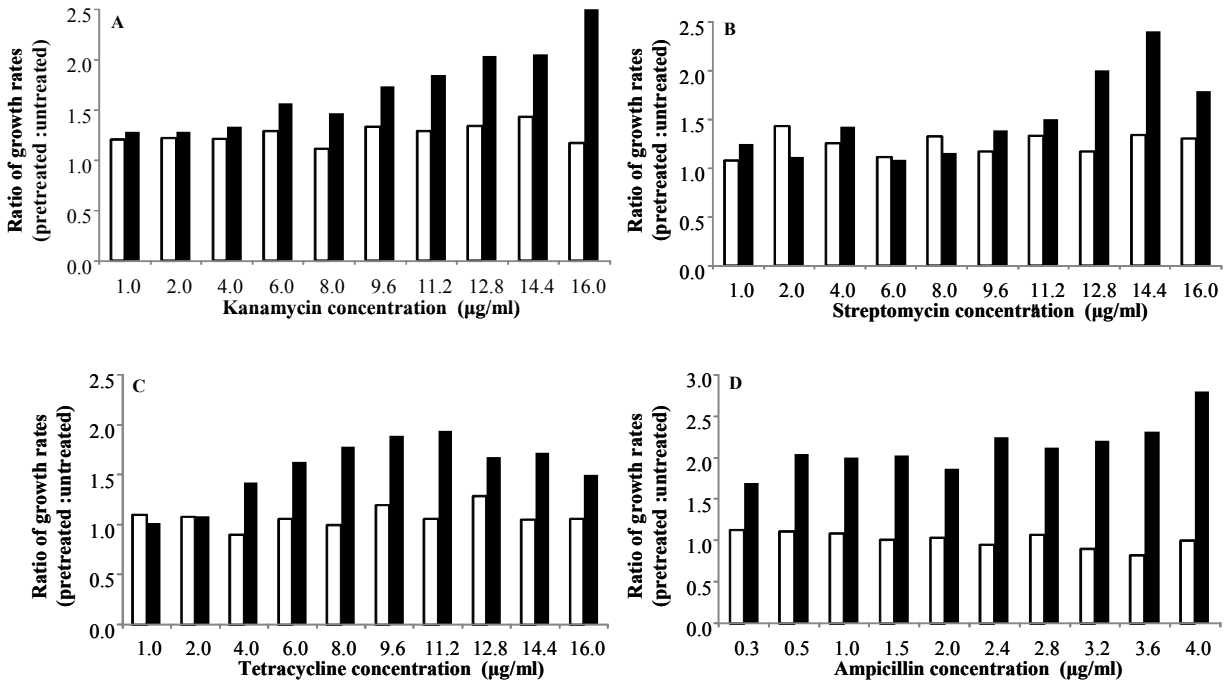

FIG. 1. Effect of kanamycin pretreatment on antibiotic resistance of *E. coli* B23 represented by the ratio of growth rate observed in pretreated and untreated cultures and subsequently challenged with (A) kanamycin, (B) streptomycin, (C) tetracycline, and (D) ampicillin. Unfilled bar represents t = 20 min, and filled bar represents t = 60 min.

ability of bacteria to limit the entry of the antibiotics. Inhibition of entry of antibiotics into the cell includes increased capsular production, outer membrane changes such as decreased porin production, and finally inner membrane restructuring. We will discuss the potential contributions of each of these aspects.

The first possible mechanism involved in the transient antibiotic resistance is the increased capsular production in response to sub-lethal level of kanamycin. Antibiotic-treatment has been shown to increase the production of soluble as well as cell bound capsular polysaccharide in bacteria (7, 11). Capsular polysaccharide has been found to hinder the inward movement of antibiotic molecules (6), and this may account for some of the observed general short term resistance induced by kanamycin pre-treatment. However, capsular polysaccharide production is probably only a minor effect compared to that of entry blockage by outer membrane (19).

The result from plate counting suggests that the sub-lethal treatment of kanamycin does not affect the viability of *E. coli* B23 cells. This also supports that capsular polysccharide production does not provide much protection against sub-lethal level of antibiotics. In addition, the potential capsular production does not significantly affect the turbidity measurement. Previous studies also suggest that microscopic examination of the cultures grown in the presence of the antibiotics did not reveal any morphological abnormalities that might lead to anomalous results (7).

Bacterial adaptation to reduce permeability of the outer membrane to antibiotics in response to sub-lethal level of kanamycin is believed to be a more promising mechanism. All the tested antibiotics enter the gram-negative bacteria via the outer membrane porins, OmpC or OmpF proteins (6, 9, 18) and thus the level of expression of these porins regulates the entry of the antibiotic molecules into the cell. Researchers have found that continued exposure to sub-inhibitory level of antibiotics selects for step by step porin-expression modification, resulting in further reduced uptake at each stage. In fact, porin-deficient mutants are shown to have complete impermeability to β-lactam (9). The absolute and relative levels of expression of the OmpF and

TABLE 1. Effect of kanamycin pretreatment on antibiotic resistance of *E. coli* B23, represented by the observed MIC in pretreated and untreated cultures. The MIC was later estimated by looking for the lowest concentration where absence of growth was observed.

Antibiotic	Minimal Inhibitory Concentration	
	Kanamycin pretreated	Untreated
Kanamycin	6	4
Streptomycin	6	0
Tetracycline	4	4
Ampicillin	1.5	1.5

OmpC genes are shown to be balanced according to internal and external conditions (12). Therefore, it is possible that *E. coli* B23, in response to exposure to sub-lethal level of kanamycin, either down-regulated the expression of OmpF and OmpC genes and/or exhibit a shift in the type of porin expressed to decrease permeability of the membrane and hence increased protection against antibiotics (16). In addition to the reduced influx via altered porin phenotypes, a simultaneous overexpression of an AraB efflux pump was observed that resulted in extrusion of incoming antibiotic molecules (15, 16). Together, these modifications can severely decrease the intracellular antibiotics concentration.

As seen in (Fig. 1) during the time course of antibiotic treatment, at t = 20 min the kanamycin pretreated *E. coli* B23 culture does not show a higher growth rate compared to the control group. The ratios between the turbidity of pretreated and untreated groups remain consistent as the levels of antibiotics used for treatment are increased. The cross resistance induced is not obvious at this initial time point since the antibiotic treatments have just started and are not yet effective. Later results obtained at t = 60 min show that although the turbidity between the pre-treated samples is very similar to the turbidity of the control group at low antibiotic concentrations, the pre-treated samples exhibit a significantly higher turbidity at high antibiotic concentrations than the control group. A possible explanation for this observation is an intrinsic bacterial adaptation to reduce uptake of antibiotics through porins as mentioned earlier.

The untreated group is expected to grow relatively well at antibiotic concentrations below MIC, as the results from our preliminary MIC determination assay have suggested. It was found that there is a sacrifice for the down-regulation of porin proteins and subsequent decrease in permeability to antibiotics—a severe loss of bacterial fitness owing to restricted uptake of nutrients (16). It is possible that when concentration of antibiotics are below the MIC, there is a balance between the loss of fitness and gain of survival due to porin-related cross resistance which yields a net growth rate that is similar to the growth rate of *E. coli* that have not been subjected to a sub-lethal treatment of kanamycin. Nevertheless, when the antibiotic concentrations were increase beyond MIC, the protection overrides the decrease in fitness and the pre-treated bacteria were shown to have a higher survival and growth rate compared to the control group. Since the ratios of pretreated to untreated increased as the concentration of antibiotics increased, the pre-treated *E. coli* B23 are shown to have better ability to adapt and to grow in presence of normally lethal doses of antibiotics.

By comparing these results to the results obtained from the control group, our hypothesis of pretreatment yielding cross-protection to killing by antibiotics was supported. In addition, the ratios started to decline as the concentration of streptomycin and ampicillin reaches 14.4 μg/ml and 12.8 μg/ml respectively. These decreases in ratio might be cut off point indicating the maximum capacity of the cross-protection elicited by the pretreatment; despite the cross-protection, the *E. coli* cells are killed regardless as the intracellular antibiotic concentration is beyond the cell's defense limit.

Although long term protection was not observed against tetracycline and ampicillin after sub-lethal level of kanamycin pretreatments, it was observed in kanamycin and streptomycin (Table 1). The difference in observation could be explained if the down regulation of porin proteins is a short term protection mechanism against antibiotics penetration. The down regulation could have resulted in severe loss of bacterial fitness owing to restricted entry of nutrients (3); therefore the porins will eventually have to be expressed again for uptake of nutrients.

A possible explanation for the long term (24 hours) resistance to kanamycin and streptomycin treatments in kanamycin-pretreated cells can have inner membrane restructuring. The aminoglycosides use Energy-Dependent Phase I (EDPI) to cross the plasma membrane and EDPI is found to vary in duration and rate depending on the external concentration of aminoglycosides (2). However, the mechanism of down regulation of aminoglycoside uptake by bacteria is not yet well understood (14, 20). Therefore, we hypothesize that a down regulation or modification of EDPI during pretreatment will inhibit the aminoglycoside uptake across cytoplasmic membrane. As a result, both EDPI dependent active uptake of kanamycin and streptomycin will be hindered and these antibiotics will no long be able to enter the cell. This long term resistance is absent from tetracycline and ampicillin as they are EDPI independent. Tetracycline use passive diffusion, once they enter periplasm through the porins (2, 18) and ampicillin does not require crossing the plasma membrane since it inhibits bacterial cell wall synthesis, which takes place in the periplasmic space. This difference between antibiotic resistance only appears in long term experiment, because down regulation of porin is transient and the protection disappears once they are restored.

In general, pretreatment with sublethal levels of kanamycin provided bacteria with a transient cross resistance against treatment of all the chosen antibiotics. In the long term, however, cross resistance was only observed against aminoglycoside treatments. This suggests that the mechanism for short term (1.5 hours) and the long term (24 hours) protection triggered by kanamycin pre-treatment is different. However, the actual mechanisms involved remain unknown and

require further research.

FUTURE EXPERIMENTS

In this experiment, the strain *E. coli* B23 was subjected to sublethal kanamycin before exposing it to lethal amounts of antibiotics. To add to our understanding of the mechanisms of cross resistance, further experiments can be focused on quantifying the amount of porins that are presented on the cell surface when a cell has been exposed to a pretreatment of kanamycin. By comparing the amount of porins on the kanamycin pretreated cells with the control group, one can test the porin hypothesis that we formulated in this experiment.

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