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Effect of Growth in Sublethal Levels of Kanamycin and Streptomycin on Capsular Polysaccharide Production and Antibiotic Resistance in *Escherichia coli* B23

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Aerobic Gram negative bacilli in general demonstrate adaptive resistance to stresses from the environment such as the ability to transiently acquire resistance against antimicrobial killing by certain antibiotics. Previous studies have shown that Escherichia coli B23 pretreated with sublethal doses of kanamycin and streptomycin showed elevated levels of capsular polysaccharide. It has also been reported that bacterial capsules may impede uptake of aminoglycosides and thus protect the cell. This study attempted to investigate the potential development of protection from the antibiotics kanamycin and streptomycin due to the elevated capsular polysaccharide production. E. coli B23 was pretreated with 10 µg/ml of kanamycin or streptomycin. It was observed that both kanamycin and streptomycin pretreated cells developed increased antibiotic resistance relative to untreated cells, which correlated with a significant increase in the production of capsular polysaccharide.

Capsular polysaccharides (CPS) found in members of the *Enterobacteriaceae* family play an important role in mediating the interaction between the bacteria and their surrounding environment (13). These extracellular polysaccharides can remain attached to the cell surface in a capsular form or be released as slime (13). *Escherichia coli* produce a wide variety of CPS termed K antigens that are further divided into capsule groups (12). In particular, group 1 capsules have been shown to be similar in structure, genetics, and expression to capsules produced by *Klebsiella pneumonia* (12). Previous studies have confirmed that a highly conserved region of DNA involved in the enzymatic production and translocation of CPS existed between these two species. Held *et al.* (5) have shown that *K. pneumonia* has increased CPS production upon treatment with sublethal doses of the antimicrobial agents ciprofloxacin and ceftazidime (5). Likewise, Ganal *et al*. (3) have demonstrated similar results by exposing *E. coli* B23 to sublethal doses of kanamycin and streptomycin (3). These studies suggest that important alterations in cell morphology and metabolic activity may occur after the exposure of bacteria to sublethal doses of antibiotics (5).

Aminoglycosides are known to bind to the 30S bacterial ribosomal subunits and in this way inhibit protein synthesis (4); these antibiotics cause the misreading of mRNA and the inhibition of peptidyl-tRNA translocation from the A-site to the P-site (9). Yet upon the exposure to aminoglycosides and possible osmotic stress, the activated expression of *cps* gene cluster in capsular polysaccharide production might be implicated as an innate, adaptive antibiotic resistance mechanism in *E. coli* (2). In addition, another common resistance mechanism in Gram negative bacilli is membrane impermeabilization that results in decreased drug uptake efficiency (5) and possibly an increased CPS production. Aminoglycoside diffusion across the cell membrane can also be impeded by an increased thickness of bacterial capsule and an increased attraction between the opposite charges of aminoglycosides and capsule constituents (4).

This experiment examined the potential correlation between the increased CPS production by sublethal kanamycin and streptomycin pretreatments and the potential increased antibiotic resistance in *E. coli* B23. Following sublethal antibiotic pretreatment, CPS was isolated and assayed for increased CPS. Investigations of antibiotic resistance were carried out by pre-exposing *E. coli* B23 to sublethal doses of kanamycin and streptomycin and then subjecting them to various concentrations of the respective antibiotics.

MATERIALS AND METHODS

Bacterial strain. *Escherichia coli* B23 was obtained from the MICB 421 culture collection in the Microbiology and Immunology Department of the University of British Columbia.

Antibiotic stock solutions. Kanamycin monosulfate (Sigma #K4000) and Streptomycin sulfate (IGN-#100556) were prepared by dissolving each antibiotic in distilled water to a final concentration of 20 mg/ml. The solutions were then filter

sterilized using a Millipore 0.2 µm GSWP nitrocellulose membrane. Sterilized antibiotic solutions were stored at -20°C.

Growth of *E. coli* B23. An overnight culture was prepared by inoculating 100 ml of M9 minimal medium (8.5 mM NaCl, 49 mM Na_2HPO_4, 22 mM KH_2PO_4, 18 mM NH_4Cl, 0.80 mM $MgSO_4$, and 0.4% (w/v) glycerol) with a loopful of *E. coli* B23. The inoculated culture was placed overnight in a shaking water bath at 200 rpm and 37°C. Twenty-five ml of the overnight culture was then inoculated into three flasks with 160 ml M9 minimal medium. The newly inoculated cultures were grown for 4 hr to 0.55 OD_{460} by incubating them in a shaking water bath at 200 rpm and 37°C.

Sublethal antibiotic pretreatment. Kanamycin stock solution was added to one inoculated 160 ml culture to create a 10 µg/ml kanamycin pretreatment condition. Streptomycin stock solution was added to the second 160 ml culture to create a 10 µg/ml streptomycin pretreatment condition. The third 160 ml inoculated culture served as a control condition. All three cultures were incubated for an additional 1 h in a shaking water bath at 200 rpm and 37°C in order to induce the potential increase in capsular polysaccharide and antibiotic resistance. Turbidity was measured after the additional 1 h incubation using Beckman DU® 530 spectrophotometer set at wavelength 460 nm, using M9 minimal medium as blank.

Antibiotic resistance test. To 18 x 120 mm glass test tubes, sufficient volumes of kanamycin stock solution were added to produce four tubes, each with final kanamycin concentrations of 0.3, 0.6, 1.3, 2.5, 5.0, 10.0, 15.0, 20.0, 25.0 and 30.0 µg/ml for 5 ml total volume. M9 minimal medium (8.5 mM NaCl, 49 mM Na_2HPO_4, 22 mM KH_2PO_4, 18 mM NH_4Cl, 0.80 mM $MgSO_4$, and 0.4% (w/v) glycerol) was added to bring the final volume to 3.8 ml. The same procedure was used to prepare 10 test tubes containing streptomycin. In duplicate, 1.2 ml of the respective bacterial cultures from the 160 ml cultures were added aseptically to corresponding test tubes; kanamycin pretreated *E. coli* B23 was added to one test tube containing kanamycin, and to another test tube with for streptomycin. Untreated *E. coli* B23 were added to test tubes containing kanamycin or streptomycin. A control was set up in duplicate by adding 1.2 ml of untreated *E. coli* B23 to 3.8 ml of M9 minimal medium that lacked both antibiotics. Samples were vortexed carefully, loaded onto a tube roller (Glas-Col Apparatus Co. #RD250), and incubated overnight at 37°C at full dial. Turbidity of the samples was read using the Spectronic20D spectrophotometer with wavelength set at 460 nm, using M9 minimal medium as blank.

Capsule isolation. Isolation of the capsular polysaccharide was performed with some modifications to the protocol outlined by Ganal *et al.* (3). 160 ml of each culture sample was centrifuged using a Beckman J2-21 centrifuge using the JA-14 rotor at 17,000 x g for 20 min. The supernatants were discarded and the residual pellets were resuspended in PBS (137 mM NaCl, 2.7 mM KCl 1.4mM Na_2HPO_4, 1.8 mM KH_2PO_4) to ¼ of the original volume. PBS resuspended pellets were blended in an Osterizer 8300 Series Waring blender for 5 min at the liquefy setting. Exopolymers were then precipitated by adding two volumes of ice-cold acetone. The resulting suspension was centrifuged using a Beckman J2-21 centrifuge using the JA-14 rotor at 6,000 x g for 10 min. The resultant supernatants were discarded and the pellet was resuspended in 10 ml of distilled water and placed in Spectra/Por® molecularporus membrane dialysis tubes with a molecular weight cut-off of 6,000-800 kDa. These samples were dialyzed against 1L of distilled water at 4°C for 24 h. The dialyzed exopolymers were dried overnight using a lyophilizer. The dried crude exopolymers were dissolved in 20 ml of sterile 10 mM $MgCl_2$. Deoxyribonuclease I (DNaseI) (Sigma #2326670) and ribonuclease A (RNaseA) (Fermentas #EN0531) were added to each dissolved sample to final concentrations of 5 µg/ml and 0.1 mg/ml, respectively. Samples were incubated for 5 h in a shaking water bath at 37°C. Pronase (Boehringer Mannheim #165921) was then added to a final concentration of 0.1 mg/ml and the samples were incubated for 24 h in a shaking water bath at 37°C. Samples were then heated to 80°C in a stationary water bath for 30 min and centrifuged in a Beckman J2-21 centrifuge using the JA-14 rotor at 17,000 x g for 20 min. The resulting supernatants were dialyzed and lyophilized as described above. The partially purified and dried exopolysaccharides were dissolved in 0.05 M Tris base containing 0.1 M NaCl. Sodium deoxycholate was added to a final concentration equal to 0.75 micellar concentrations (approx 1.5 mM). Sample mixtures were incubated at 65°C for 15 min, chilled to room temperature on ice and then 20% acetic acid was added to final concentration of 1%. Lipopolysaccharides (LPS) and deoxycholate were pelleted off by centrifuging in a Beckman J2-21 centrifuge using the JA-14 rotor at 16,000 x g for 5 min. Purified capsule contained in the supernatants was stored at 4°C until use.

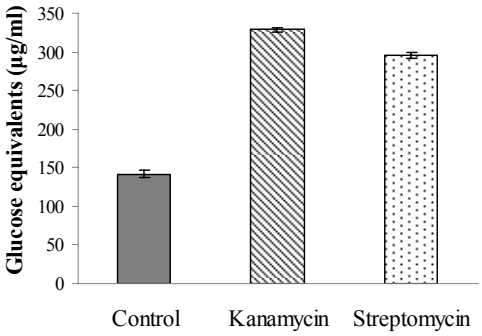

FIG. 1. The effect of antibiotic treatment on the total capsular polysaccharide in *E. coli* B23. Kanamycin and streptomycin samples refer to cells pretreated in a condition of 10 µg/ml of kanamycin and streptomycin respectively for 1 h. Control cells were left untreated. Error bars established for duplicate readings of each sample.

Anthrone carbohydrate assay (4). Anthrone reagent was prepared by dissolving 200 mg of anthrone (MC&B-#AX1655) in 5 ml of absolute ethanol. The solution was then made up to 100 ml using concentrated (93%) sulfuric acid (JT Baker) and chilled until used. Duplicate of half millilitre samples of each purified capsule sample were placed into glass test tubes and chilled on ice for 2 to 3 min. Two and one half ml of anthrone reagent was added to the samples in the chilled test tubes, and carefully vortexed. The tubes were then transferred to a boiling (100°C) stationary water bath and capped with glass marbles. Tubes were incubated for exactly 10 min and then transferred back to ice to cool and stop the reaction. Once cooled, 3 ml volumes of samples were read at 625 nm using a Spectronic20D spectrophotometer. Glucose equivalent standards ranging from 0 µg/ml to 100 µg/ml were prepared using α-D(+) glucose (Sigma #G-5000) diluted with M9 minimal medium without glycerol. Standards were treated the same way as test samples, and a standard curve was generated.

RESULTS

Anthrone carbohydrate assay. The total carbohydrate concentration from the isolated capsule increased in antibiotic pretreated *E.*

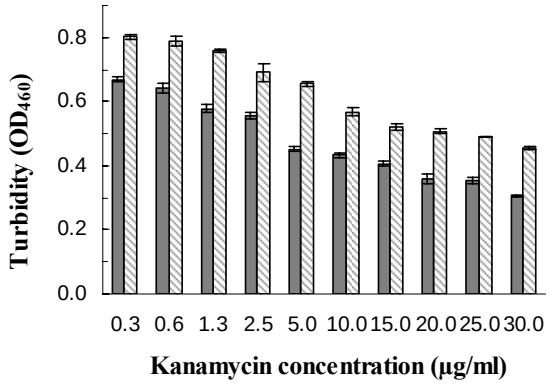

FIG. 2. Effect of kanamycin pretreatment on *E. coli* B23 as a representation of antibiotic resistance. Sample cells (dashed pattern) were pretreated in a condition of 10 µg/ml of kanamycin for 1 h, and control cells (solid grey) were left untreated. All cells were subsequently challenged with varying concentrations of kanamycin antibiotics, and grown in M9 minimal medium at 37°C with mild aeration. Error bars established for duplicate readings of each sample.

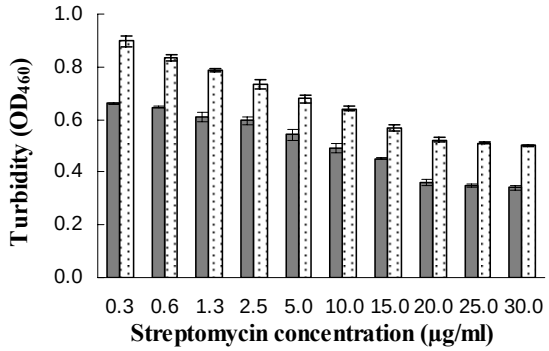

FIG. 3. Effect of streptomycin pretreatment on *E. coli* B23 as a representation of antibiotic resistance. Sample cells (dotted pattern) were pretreated in a condition of 10 µg/ml of streptomycin for 1 h, and control cells (solid grey) were left untreated. All cells were subsequently challenged with varying concentrations of streptomycin antibiotics, and grown in M9 minimal medium at 37°C with mild aeration. Error bars established for duplicate readings of each sample.

coli B23 (FIG. 1). Compared to the control, the total carbohydrate measured in the anthrone assay was ~2.4 times higher in kanamycin pretreated cells and ~2.2 times higher in streptomycin pretreated cells.

Antibiotic resistance test. Figures represented the effects of pretreatment using aminoglycosides and the subsequent effect on antibiotic resistance observed through cell growth. In general, it was observed that untreated cells showed lower growth and less resistance compared to the pretreated cells at the same antibiotic concentration. More specifically, untreated *E. coli* B23 that grew without any antibiotic treatment had an OD_{460} reading of ~1.20, the highest turbidity from all the other sampled tubes; implicitly, this cell concentration was ~1.4 times higher than pretreated cultures (FIG. 2 and 3) and ~1.8 times higher than the untreated control cultures that were subsequently exposed to the lowest concentration in antibiotic treatment (FIG. 2 and 3). Since all cultures were established with an initial turbidity of 0.20 OD_{460}, a dosage-dependent effect was observed for both aminoglycoside pretreated and untreated cultures; increasing antibiotic concentrations showed decreased cell growth for both pretreated and untreated cells. Moreover, a consistent trend of higher turbidity for pretreated cells compared to untreated cells was observed across all the different concentrations of kanamycin and streptomycin (FIG. 2 and 3). Furthermore, streptomycin pretreated cells grew to a higher turbidity than kanamycin pretreated cells across all the respective antibiotic concentrations. Streptomycin pretreated samples increased turbidity by 28 to 47% in the antibiotic test. For kanamycin pretreated samples, the turbidity increased 29 to 45%.

DISCUSSION

The total capsular polysaccharide results (FIG. 1) showed that *E. coli* B23 pretreated with either aminoglycoside produced significantly higher levels of CPS compared to the control cells without pretreatment. This was not a surprising finding as Ganal *et al.* (3) had demonstrated this result previously using a similar approach (3). Further support for this observation could be found in an analogous experiment by Held *et al.* (5) who demonstrated an increase in CPS and capsule diameter of *K. pneumonia* upon exposure to sublethal doses of the antibiotics ciprofloxacin and ceftazidine (5). Moreover, the *cps* gene cluster encodes several of the enzymes involved in the synthesis and translocation of CPS, and is highly conserved between several species of *E. coli* and *K. pneumonia* (12). The observation of insertion sequence (IS) elements, present upstream in the *cps* cluster region of both species implicates that there was a likely transfer of the *cps* locus between the organisms (12). The striking similarities in the structure and genetics of the extracellular capsule between these two species provide a strong support that *K. pneumonia* genes are likely transferable to *E. coli*. Studies have demonstrated that the exposure of bacteria to sublethal doses of antibiotic could potentially result in a significant

alteration in metabolic activity (5). The differences in growth patterns were speculated to be a result of cellular energy and resource diversion for CPS up-regulation, which was observed in the total carbohydrate assay. A growth curve experiment was attempted to characterize the growth patterns between aminoglycoside pretreated samples and the untreated controls challenged with the same antibiotic concentration. However, the growth curve was not included in our analyses because the data were collected for only a short period of time. Ideally, the growth curve experiment should coincide with the duration of the antibiotic resistance test in order to track the analogous growth pattern of the latter. In that case, it would be expected that at some point after the antibiotic pretreatment, pretreated samples would have a slower growth rate than the untreated control culture because of more CPS production. Nevertheless, the pretreated samples would eventually outgrow the untreated control culture at a later point in time.

Results from the total carbohydrate assay revealed that ~100 times more capsular polysaccharide was isolated from all three (kanamycin, streptomycin, and untreated control) samples as compared to results presented by Ganal *et al.* (3). These quantitative differences could be due to changes in the methods to improve measurement accuracy and recovery. The speed setting selected for the Waring blender used to extract exopolysaccharide had been lowered but the blending time was extended 5 min longer to augment the slower blending speed. As a result, sample loss due to evaporation and residue adherence onto the blender wall was greatly reduced. The extended blending time might have also increased the likelihood of more bacterial capsules being effectively separated from the cells. The volume of NaCl solution used to dissolve the exopolysaccharide had been halved, so that the absorbance reading at 625nm could be obtained within 0.1 and 0.6 OD_{625} units, which was the accurate range of measurement for the Spectronic20D spectrophotometer. The trend presented by Ganal *et al.* (3) was still able to be replicated, in which CPS levels of kanamycin pretreated cells produced ~1.1 times more CPS than streptomycin pretreated cells. However, this did not match the expectation that greater CPS production would result in better resistance to aminoglycosides; greater resistance was observed in streptomycin pretreated cells (FIG. 2 and 3).

The increase in capsular polysaccharide might possibly not have been a direct result of the antibiotic treatment. Aminoglycoside pretreated cells had a less stable membrane structure (14) and so capsular polysaccharide could be dislodged easier upon blending. There was no evidence in this experiment to distinguish whether there was an actual increase in capsular polysaccharide synthesis or that increase observed was due to decreased membrane structural integrity. The total carbohydrate content of all the treatment groups should be measured and compared to distinguish between these two possibilities. If there was a significant increase in total carbohydrate in pretreated cells compared to control cells, then this would indicate that additional carbohydrates were synthesized in response to the antibiotic pretreatment.

E. coli B23 pretreated with sublethal dose of kanamycin or streptomycin were more resistant to antibiotic challenge compared to control cultures (FIG. 2 and 3). Increased resistance was defined with significantly higher turbidity (OD_{460}) readings for antibiotic pretreated cultures compared to controls when both were subjected to identical antibiotic challenge and subsequent growth conditions. This finding was consistent to the literature; aerobic Gram negative bacilli have shown adaptive resistance to transiently reduce microbial killing in the original susceptible bacteria (10). This resistance mechanism involves membrane impermeabilization as a mean to reduce antibiotic uptake and the subsequent accumulation inside the cell (10). Since both kanamycin and streptomycin pretreated cells showed a similar acquired resistance to the respective antibiotic after pretreatment, the results supported the notion of non-specific stress response to aminoglycosides. Although the mechanisms by which kanamycin and streptomycin inhibit protein synthesis are slightly different, the ultimate antimicrobial effect on the cells is similar; with physiological stress imposed, the cells are stimulated to respond and adapt in similar ways. Although the expression of specific aminoglycoside-modifying enzymes is a possible mechanism behind the observed resistance, it is less likely in this case as the observed resistance showed a similar trend for both kanamycin and streptomycin treated cells.

Although a causative relationship between increased CPS and increased antibiotic resistance was not proven, a correlation existed between these two observations. Previous literature has provided a strong basis for the observation that pretreatment of bacteria with sublethal doses of aminoglycosides may cause an increase in CPS production (FIG. 1). Previous research indicated that aminoglycoside treatment could impair the

membrane integrity of bacteria; with the inhibition of protein synthesis by aminoglycoside, a significant proportion of structural membrane proteins would decline (14). Previous studies also showed that one of the signaling pathways for activating the expression of the *cps* gene cluster appeared to act in response to environmental stimuli such as osmotic stress (2). Osmotic stress may be a result of reduced membrane integrity, which could trigger the observed increase in CPS production. Increased CPS may lead to greater bacterial membrane impermeabilization, which would confer resistance of decreased antibiotic uptake and accumulation (10).

Since the percentage increase in the turbidity of antibiotic pretreated cells compared to the turbidity of untreated cells was quite similar across all concentrations, the difference in turbidity measurements between the pretreated and untreated samples could possibly be due to the additional light scattering from the increased exopolysaccharides in the extracellular milieu. Since this possibility was not eliminated, it would be worthwhile to repeat this experiment with a phase contrast microscope to do cell counts. If there was indeed a difference in growth due to a difference in cells counted between pretreated and untreated samples, then the possibility of CPS being responsible for the difference in turbidity observed between pretreated and untreated samples would be eliminated.

The percentage increase in the turbidity of pretreated cells from untreated cells differed depending on the antibiotic concentrations used to challenge the bacteria. There seemed to be a threshold difference between 15.0 µg/ml and 20.0 µg/ml of antibiotic concentrations in the response of pretreated *E. coli* B23 to antibiotics. These results showed that pretreated *E. coli* B23 challenged with kanamycin or streptomycin concentrations between 20.0 µg/ml and 30.0 µg/ml displayed ~1.5 times increase in growth compared to untreated control cells between 0.3 µg/ml and 15.0 µg/ml. Therefore, exceeding a certain threshold of antibiotic concentration might elicit a more robust resistance response, which allowed for increased growth. It was also observed that streptomycin pretreated cells had a significantly greater resistance across all streptomycin concentrations than kanamycin pretreated cells challenged with the different concentrations of kanamycin.

E. coli B23 pretreated by sublethal doses of kanamycin or streptomycin manifested increased resistance to the respective antibiotic treatment. Moreover, the same pretreated cells increased their CPS production. Thus, there is a significant correlation between observed increased CPS and increased antibiotic resistance to streptomycin and kanamycin. Kanamycin and streptomycin pretreatment has been shown to increase antibiotic resistance in *E. coli* B23, but it is not clear whether the effect is a general increase in resistance or a more specific effect.

FUTURE EXPERIMENTS

A more complete analysis needs to be conducted for cross reactivity by testing kanamycin pretreated cells with streptomycin and streptomycin pretreated cells with kanamycin; this would assess whether the effect is antibiotic specific or a more broad based resistance. It would also be worthwhile to know if aminoglycosides pretreatment can show increased antibiotic resistance in cells with other groups of antibiotics such as β-lactams and tetracycline.

Linking the correlation between the increase in CPS production with the increase in antibiotic resistance to aminoglycosides as causative effect can be accomplished with a *cps*-deleted *E. coli* B23 mutant. A causative relationship is established when the effect of increased antibiotic resistance is shown in pretreated cells, but not observed in the *cps*-deleted mutants.

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