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Persistence of Antibiotic Resistance and Capsule in *E. coli* B23 after Removal from Sublethal Kanamycin Treatment

Henry Liu, Mei Zhu and Shawn Zhu
Department of Microbiology & Immunology, University of British Columbia

Kanamycin belongs to the aminoglycoside class of bacteriostatic antibiotics that inhibits protein synthesis. Previous studies have shown that treatment with sublethal levels of kanamycin causes the extracellular polysaccharide capsule in *E. coli* B23 to increase in size, and potentially confers the bacterium with kanamycin resistance. This study attempted to confirm this by establishing that increased capsule size is the cause of kanamycin resistance by looking at the correlation between the increase in capsule size and the kanamycin resistance. This correlation was investigated by monitoring the change in capsule size and the kanamycin resistance of pretreated *E. coli* B23 after it was transferred back to antibiotic-free M9 minimal medium. The degree of kanamycin resistance was found to be directly related to the capsule content of the cell. Immediately after removal from pre-lethal levels of kanamycin, the cells were resistant and had high levels of capsule. After 2.5 h, pretreated cells began to lose capsule and kanamycin resistance simultaneously.

Kanamycin is a bactericidial antibiotic belonging to the aminoglycoside family, and inhibits protein synthesis by preventing translocation of peptidyl-tRNAs from A-site to the P-site on the 30S subunit of ribosomes (5). Previous experiments have found that treating *E. coli* B23 with sub-lethal levels of kanamycin leads to increased production of capsule (2) and increased resistance to kanamycin (7).

E. coli capsules are extracellular polysaccharides, with colanic acid as its main component (3). The increased resistance to kanamycin could be due to the colanic acid of the capsule preventing the uptake of kanamycin (4, 5). One way this may be achieved is due to the increased thickness in the capsule. Another possibility is the opposite charge attraction between the capsule itself and kanamycin, thereby hindering the movement of kanamycin (4). If the capsule of *E. coli* B23 persists after removal from sub-lethal levels of kanamycin, the cells would be more resistant to lethal levels kanamycin due to the exclusion effect conferred by the increased size of capsule.

Our experiment investigated the direct link between capsule production and kanamycin resistance. We proposed that the capsule gained after pretreatment would not be persistent, and its kanamycin resistance would be lost in parallel to the loss of capsule. This may be investigated by looking at the co-relationship between capsule size and kanamycin resistance. To hold true, kanamycin resistance and capsule size would co-vary in a positive correlation. In other words, if the capsule persisted, kanamycin resistance would also remain; if the capsule size returned to original state, *E. coli* would no longer be resistant to kanamycin.

MATERIALS AND METHODS

Capsule isolation. Isolation of the capsular polysaccharide was performed with some modifications to the protocol outlined by Lu *et al*. (1). Culture sample was taken before pretreatment, right after pretreatment (T0), and also 1, 2.5, and 24 hours after pretreated cells were transferred back to kanamycin-free M9 minimal media (T1, T2.5, and T24 respectively). 70ml of each culture sample was centrifuged at 17,000 x g for 20 mins using a Beckman J2-21 centrifuge with a JA-20 rotor, and the residual pellets were resuspended in PBS. PBS resuspended pellets were blended for 5 min using a 1107900 series Bead-beater on maximum setting. Blended suspension was centrifuged at 10,000 x g for 15 mins using a Beckman J2-21 centrifuge with a JA-20 rotor, and the residual cell pellets were removed. Exopolymers were then precipitated by adding two volumes of ice-cold acetone to remaining supernatant. The resulting suspension was centrifuged at 6,000 x g for 10 min. The pellet was resuspended in 10 ml of distilled water and placed in 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¡ãC for 48 hours with the dialysis water changed once. The dialyzed exopolymers were dried overnight using a lyophilizer. The dried crude exopolymers were dissolved in 20ml 10mM $MgCl_2$. Deoxyribonuclease I (Dnase I) and ribonuclease A (Rnase A) were added to each dissolved sample to final concentrations of 5 ¦Mg/ml and 0.1 mg/ml, respectively. Samples were incubated for 5 hours in a shaking water bath at 37˚γC. Pronase 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¡ãC. Samples were then heated to 80¡ãC for 30 min and centrifuged at 17,000 x g for 20 min using a Beckman J2-21 centrifuge with a JA-20 rotor. The resulting supernatants were dialyzed against 4L of distilled water for 24 hours, 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 15mM. Sample mixtures were incubated at 65¡ãC for 15 min, chilled to room temperature on ice and then acetic acid was added to final concentration of 1% v/v. LPS and deoxycholate were pelleted off by centrifuging 16,000 x g for 5 min. Purified capsule contained in the supernatants was stored at 4¡ãC until use.

Anthrone carbohydrate assay. (11) - Anthrone reagent was prepared by dissolving 200 mg of anthrone in 5 ml of absolute

ethanol. The solution was then made up to 100 ml using concentrated (93%) sulfuric acid and chilled until used. Duplicate of 0.5ml purified capsule samples were placed into glass test tubes and chilled on ice for 2 to 3 min. 2.5 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¡ãC) stationary water bath and capped with glass marbles. Tubes were incubated for 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 spectrophotometer. Glucose equivalent standards ranging from 0 ¦Mg/ml to 100 ¦Mg/ml were prepared using ¦A-D(+) glucose diluted with distilled water. Standards were treated the same way as test samples, and a standard curve was generated.

Kanamycin-resistance test. Kanamycin stock was added to tubes containing 3 ml M9 minimal medium with final kanamycin concentrations of 50 ¦Mg/ml. Culture sample was taken before pretreatment, right after pretreatment (T0), and also 1, 2.5, and 24 hours after pretreated cells were transferred back to kanamycin-free M9 minimal media (T1, T2.5, and T24 respectively). In duplicate, 1.0 ml of the respective bacterial cultures from the 160 ml cell cultures were added to those kanamycin-containing test tubes; A control was set up in duplicate by adding 1.0 ml of untreated *E. coli* B23 to 4 ml of M9 minimal medium that lacked kanamycin. Samples were vortexed carefully, loaded onto a tube roller, and incubated overnight at 37¡ãC. Turbidity of the samples was read at 460 nm, using M9 minimal medium as blank.

RESULTS

Kanamycin resistance assay. With a one-hour pre-treatment of 10 µg/ml of kanamycin, *E. coli* B23 survived much better when subjected 50 µg/ml of kanamycin. At the time of sampling event, both pre-treatment (PT) and control grew to an equal turbidity in M9 minimum media at 37 °C. However after overnight exposure to 50 µg/ml of kanamycin, PT showed at least 40% better survival compared to the control (Fig. 1). Percent survival after overnight (ON) incubation in M9 media containing 50 µg/ml of kanamycin was seen as a reflection of resistance to kanamycin as shown in Figure 1. The survival for PT and control were comparable before pre-treatment. After 1 hour of initial incubation however, survival of control dipped sharply and decreased lower and lower to the eventual 19% at T=24. PT survival remained well above 90% for the first 2.5 hours. Although PT survival also decreased with time, it still held at 68%, 24 hours after being transferred back to fresh M9 media (Fig. 1). It is evident that with pre-treatment, *E. coli* B23 maintained a significantly higher resistance against kanamycin than control ($P<0.005$). This observation was expected as it was in agreement with previous studies (7)

Anthrone carbohydrate assay. With a one-hour pre-treatment in 10 µg/ml of kanamycin, *E. coli* B23 produced significantly more capsular carbohydrate as detected by anthrone carbohydrate assay using a glucose standard. Without pre-treatment, the control maintained a low, background level of capsular carbohydrate (around 15% of the PT capsule content). PT showed a 12-fold increase in capsular carbohydrate content after the 1-hour pre-treatment. A steady decline followed PT culture being transferred to fresh M9 at T=0. The control showed very minimal increase in its capsular carbohydrate content per 70 ml of culture (Table 2). The two cultures were also multiplying throughout the 24-hour period, when taken into account the number of cells, capsular carbohydrate content data showed more interesting and telling trends.

TABLE 1. Effect of 1 hour pretreatment of *E. coli* B23 with 10µg/ml of kanamycin on carbohydrate content of culture, sampled at each time point: before pretreatment (PT), after 1 hr pretreatment (this time was set to be T=0, when the pre-treated sample was transferred to antibiotic-free M9), T=1, T=2.5, T=18 and T=24. Measurement was done using anthrone carbohydrate assay.

Time Point	PT carbohydrate (ng/ml)	Control carbohydrate (ng/ml)
Before PT	17.1	18.6
T=0	216	14.3
T=1	196	21.0
T=2.5	186	23.4
T=18	144	22.1
T=24	134	33.3

After dividing the actual carbohydrate content by the culture concentration measured by OD_{625}, on a per OD_{625} basis, the change in PT capsular carbohydrate content is much more obvious. A similar increase (6 fold) was seen after pre-treatment, but the decline is much more noticeable after adjusting the raw data on the basis of OD_{625} readings. The decrease was rapid in the first 2.5 hours: 30% of the capsule content at T=0 was lost each hour after T=0. The decline slowed down markedly after 2.5 hours, losing only 22% of T=0 capsule content from T=2.5 to T=24. At T=24, the capsule content level per OD_{625} unit was only 35% more than that of the resting level, before pre-treatment. For the control, capsular carbohydrate content decreased after the first hour then remained minimal until T=24 (Fig. 2).

A parallel was seen between kanamycin resistance (Fig. 1) and capsular carbohydrate content (Fig. 2). When the culture had high resistance, it also had higher-than-normal carbohydrate content; when the culture had low resistance, it had near resting-level of capsule (barring the time point before treatment).

DISCUSSION

The cell growth and isolated capsule turbidity reading results (Fig. 1 and 2) right after sub-lethal dose of kanamycin pretreatment (time 0) confirmed the previous findings that sub-lethal doses of kanamycin

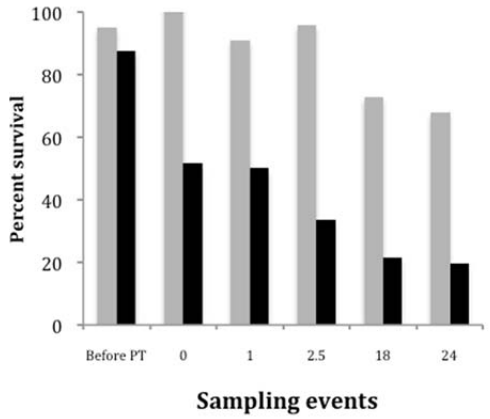

FIG. 1. Effect of 1-hour pre-treatment of *E. coli* B23 with 10 µg/ml of kanamycin on the sensitivity to subsequent exposure to 50 µg/ml of kanamycin. Pretreatment (in grey) and control (in black) were sampled at time points indicated: 0 denotes the time right after 1 hour pretreatment where pretreated sample was transferred to kanamycin-free M9; subsequent numbers denote hours after T=0. Samples were incubated overnight in M9 media and 50 µg/ml of kanamycin. Survival in terms of culture optical density (OD), as compared to the OD readings at the time of sampling, were recorded. All cultures were grown in 37 °C with mild aeration.

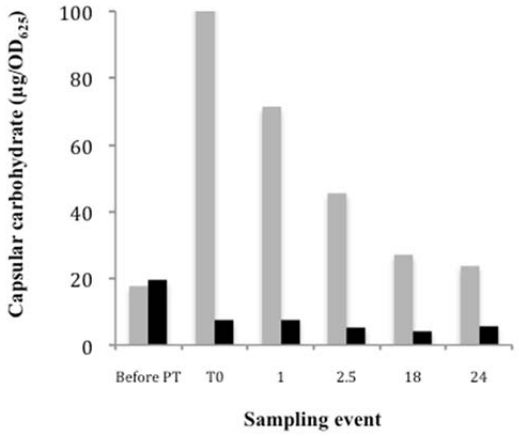

FIG. 2. Effect of 1-hour pre-treatment with 10 µg/ml of kanamycin and subsequent growth in kanamycin-free M9 on *E. coli* B23 capsular carbohydrate content, per OD_{625} unit. Pretreated sample (in grey) and control (in black) had similar levels of capsular carbohydrate before pretreatment; after 1 hour of pretreatment in 10µg/ml kanamycin, pretreated cells were transferred to kanamycin-free M9. This time was set to be T=0, subsequent numbers indicate hours after T0. All cultures were grown at 37 °C with mild aeration.

induced significantly higher levels of capsule production and also the resistance to subsequent lethal doses of kanamycin in *E. coli* B23 cells (7). In this experiment, the correlation between the increase in capsule size and the kanamycin resistance was further confirmed and elucidated by monitoring the change in capsule size and the kanamycin resistance of pretreated *E. coli* B23 after transferred back to antibiotic-free M9 minimal medium.

Figure 1 showed that E. *coli* B23 pretreated with sub-lethal dose of kanamycin maintained significantly higher levels of resistance to lethal dose of kanamycin within 2.5 hours after pretreated *E. coli* B23 were transferred from media containing kanamycin to kanamycin-free M9 minimal media (media inversion), compared to the control cells without pretreatment. At 24 hours after media inversion, pretreated *E. coli* B23 were still significantly more resistant to lethal kanamycin than the control, but the resistance was decreased overtime. Changes in gene regulation may enable a bacterium to gain resistance to certain antibiotics (11), and the exposure to sub-lethal doses of kanamycin might stimulate the expression of the gene that protect E. *coli* B23 cells against antibiotics. Without the stimulation, the protective genes will not be expressed, or those genes will be expressed in minimal amount, which could lead to the lose of antibiotic resistant ability. This is consistent with the observation that kanamycin resistance was decreasing overtime. It is very likely that after transfer back to M9 minimal media, pretreated E. *coli* B23 cells would eventually lose the gained kanamycin resistance, and revert to have the same level of resistance as the control cells.

Table 1 showed a slight increase in total capsule saccharides content of the control cells, but on a mass basis this increase was negligible. Thus the slight increase could be due to the cell growth of the control cells. Table 2 also showed the total capsular saccharides per ml of pretreated *E. coli* B23 cell culture were decreasing overtime after media inversion. The total capsular saccharide content held at a higher level within 2.5 hours after media inversion, and after the 2.5 hours it was decreased. A similar trend held when the specific capsular saccharides per OD_{625} is examined (fig. 2). The trend of capsule size change was consistent with the trend of kanamycin resistance change after transferring pretreated *E. coli* B23 cells back to kanamycin-free M9 media, which suggested a strong co-relationship between those two factors. This was consistent with previous observation that *E. coli* B23, which had thinner capsules and lower detectable levels of capsular polysaccharide, were more susceptible to kanamycin (6).

It can be hypothesized that the exposure to sub-lethal doses of kanamycin leads to the over-expression of capsule gene, and that resulted in an increase in capsule size which gave cells more resistance against subsequent lethal doses of kanamycin. It showed in previous research that aminoglycoside like kanamycin

can inhibit protein synthesis in bacteria, which could lead to the decline in significant proportion of structural membrane proteins and impair membrane integrity of bacteria (12). Previous research also indicated that environmental stimuli such as osmotic stress could induce the over-expression of *rcs*A gene, which positively regulate the transcription of capsule (*cps*) genes in *E. coli* cells (1). Thus, the reduced membrane integrity of cells may cause the osmotic stress, which stimulates over-expression of *rcs*A gene and capsules. It has been suggested that bacterial capsules prevent uptake of aminoglycosides to confer resistance and protect the cell (8). This is consistent with the observation that cell capsular saccharides and kanamycin resistance decrease over time after media inversion, because after removing sub-lethal kanamycin, cultures regained protein synthesis capacity, so they have normal cell membranes, lose the osmotic stress, thus get the normal expression of *rcs*A gene, and normal capsule size.

In conclusion, *E. coli* B23 cells pretreated with sub-lethal doses of kanamycin gained significantly higher levels of capsule production and also the resistance to subsequent lethal doses of kanamycin, and then both capsular saccharides OD_{625} per of the cell culture and kanamycin resistances were decreasing over time after media inversion. This confirmed the strong co-relationship between the increase in capsule size and the kanamycin resistance of *E. coli* B23.

FUTURE EXPERIMENTS

A future experiment looking at more isolated effect of capsule on kanamycin resistant would be helpful. Capsule production can be induced by subjecting *E. coli* B23 to osmotic stress, and subsequently have its kanamycin resistance measured. By doing so, the effects have having prior exposure to antibiotics is eliminated, so that the effect is purely increased capsule production, and not some unknown factors caused by exposure to sublethal kanamycin. The effect of a complete lack of capsule can also be tested to further isolate the effects of capsule. This can be accomplished using chemical agents to inhibit the production of capsule. The expected results of subjecting cells to osmotic stress are increased capsule size and increased kanamycin resistance, while the expected result of cells that were chemically inhibited from producing capsule is decreased kanamycin resistance. These findings would support the causative relationship between capsule and kanamycin resistance.

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