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Secondary effects of streptomycin and kanamycin on macromolecular composition of *Escherichia coli* B23 cell

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Streptomycin and kanamycin are members of the aminoglycoside antibiotic family, which are recognized as protein inhibitors. It has been previously reported that the presence of these antibiotics increased protein/carbohydrate concentrations in *Escherichia coli* B23 cells. However, this observation of increased protein concentrations would be inconsistent with the particular functions of the antibiotic treatments. Therefore, this study investigates the possibility of secondary effects of streptomycin and kanamycin on carbohydrate production. *E. coli* B23 cultures were treated with streptomycin and kanamycin and then incubated for an hour. In comparison to the control, streptomycin and kanamycin cultures both increased in total carbohydrate concentrations and decreased in protein concentration. Furthermore, this investigation attempted to identify possible carbohydrates affected by these antibiotics. Although 6-deoxyhexose and heptose concentrations decreased in the presence of streptomycin and kanmycin, hexose concentrations appeared to increase.

Aminoglycosides are well known antibiotics that inhibit protein synthesis of prokaryotic translation. Although each type of aminoglycoside may vary in the mechanism of translation inhibition, the aminoglycoside family is recognized to cause misreading of the genetic code (8). During protein translation, the mRNA is decoded by the ribosomes, but this decoding process is disrupted when aminoglycosides interact with specific sites of the 16s rRNA (8).

In addition to inhibition of protein synthesis, aminoglycosides have secondary effects which modify the macromolecular composition of growing cells. Past studies have noted that bacteria can excrete an excess of polysaccharides in the presence of antibiotics (5, 11). The over production of exopolysaccharides functions as a protective barrier for the bacteria against the natural host immunity or the antibiotic environment (6, 9).

Based on the findings of prior research (4), aminoglycoside antibiotics can inhibit overall cell growth and alter macromolecular composition by changing corresponding protein and carbohydrate levels in the cell. In particular, kanamycin and streptomycin have been found to have a significant impact on the macromolecular composition of *Escherichia coli* B23 (4). Although the antibiotic concentrations were noted to be too low for growth inhibition, the study (4) discovered an unanticipated increase of total protein/carbohydrate concentrations. However, the use of ^{14}C radioactive labelling measured the protein and carbohydrate composition in combination (4). Thus, the experiment was unable to determine if kanamycin and streptomycin directly increased protein synthesis or carbohydrate production.

Since aminoglycosides are known to inhibit protein synthesis by codon misreading, the increase of protein/carbohydrate content in the previous study was expected to be due to an increase in only carbohydrate production. Therefore, this study attempted to determine the types of sugars responsible for the increase in carbohydrate concentration.

MATERIALS AND METHODS

***Escherichia coli* B23 cultivation.** *E.coli* B23 was obtained from the MICB 421 laboratory stock from the Microbiology and Immunology Department at the University of British Columbia.

Growth and antibiotic treatment of *E. coli*. Loop of *E.coli* B23 was inoculated in 12 ml of M9 minimal medium (8.4mM NaCl, 48mM Na_2HPO_4, 22mM KH_2PO_4, 18mM NH_4Cl, 0.80mM $MgSO_4 \cdot 7H_2O$, and 0.4% (w/v) glycerol) and place into a shaking water bath at 37˚C to prepare an overnight culture. Five millilitres of an *E. coli* B23 overnight culture grown at 37 degrees Celsius was used to inoculate 100 ml of the M9 minimal medium. This culture was incubated with shaking 37˚C water bath for 3.5 hours. Three 30 ml aliquots of this culture were then split into separate flasks. Six hundred micrograms of streptomycin sulfate (Sigma S6501 – 50G) was added to create a 20 ug/ml streptomycin condition in one flask. Eight hundred micrograms of 75% (w/w) kanamycin monosulfate from *Streptomyces kanamyceticus* (Sigma K-4000) was added to create a 20 ug/ml kanamycin condition in another flask. The third flask did not contain any antibiotics and served as a control condition. These 3 cultures were grown in a shaking 37˚C water bath for 1 hour. Turbidity measurements were taken with a spectrophotometer (Spec.20) at OD_{460} at the initial inoculation of culture, at the end of the 3.5 hour incubation, and at the end of the 1 hour antibiotic treatment incubation. It was assumed that an OD_{460} unit was equivalent to a cell concentration of $5x10^8$ cells/ml.

Preparation of cell lysate. A modified Park-Hancock fractionation procedure was used to isolate the outer cell peptides and carbohydrates of *E. coli* (7). Cultures were centrifuged for 10 minutes at 7000 rpm and 15˚C. The supernatants were decanted and the pellets resuspended in 2 ml of distilled water. Centrifugation was repeated and the pellets were resuspended in 2.5 ml of chilled 25% (w/v) trichloroacetic acid (TCA) solution and left to sit for 10

minutes in an ice bath. Samples were then centrifuged at 7000 rpm for 10 minutes at 0˚C. The resulting pellets were resuspended in 2.5 ml of 75% (v/v) ethanol and left to sit at room temperature for 10 minutes.

Protein-carbohydrate isolation. The lysates were centrifuged at 7000 rpm at 15 degrees Celsius for 10 minutes. Pellets were resuspended in 2.5 ml of a 5% (w/v) TCA solution and heated in a boiling water bath for 5 minutes. The lysates were left to cool to room temperature and then centrifuged at 7000 rpm for 10 minutes at 15˚C. Pellets were resuspended in 1 ml of distilled water.

Chemical Assays. Resulting fractionated cell lysates were tested for the amount of total protein described by the quantitation method by Bradford (1)**.** The Bradford Assay quantitates protein depending on measuring changes in absorbance at a wavelength of 595nm when Coomassie brilliant blue G250 interacts with protein. Chicken egg albumin (from MICB 421 lab) was used as a standard to determine protein concentrations. Secondly, an anthrone total carbohydrate assay was done on the resulting fractionated cell lysates, testing for the amount of total carbohydrate by the anthrone reaction (3)**.** The anthrone reaction consists of sugars reacted with the anthrone reagent to produce a blue-green color. The samples were then mixed and boiled with sulphuric acid to complete the reaction. Two hundred microlitres each of the fractionated cell lystates were tested for the amount of hexose by the hexose sugar assay stated in (5). As well, another 200 ul from each of the fractionated cell lystates were tested for the amount of 6-Deoxyhexose by the 6-Deoxyhexose sugar assay (5). A third set of 200 ul from each of the fractionated cell lysates were tested for the amount of heptose by the Heptose sugar assay (5). The hexose, 6-deoxyhexose, and the heptose sugar assay all have similar preparations, which include mixing and boiling the samples with sulphuric acid, addition of cysteine hydrochloric acid, and an incubation step in the dark. Main differences were that the hexose assay only required a 30 minute incubation compared to 3 hours for the other two, and that each assay measured the difference between different wavelengths: 350 and 600nm in the hexose, 396 and 427 in the 6-deoxyhexose, and 500 and 545 in the heptose assay.

India ink stain. Samples were taken after 1 hour incubation to test for the presence of capsules. Sample was air-dried on slides, then heat fixed. Crystal violet stain was added to stain the cells for 1 minute, and then washed off with distilled water. India ink was then added to stain the capsules for one minute. Samples were viewed with a light microscope under 100x oil immersion.

RESULTS

Total protein concentration. Total protein did not increase in antibiotic treated samples. According to the Bradford Assay, the protein concentration for the control sample was the highest, while the streptomycin treated sample was the 2nd highest and kanamycin treated sample was the lowest (Fig 1 and Table 1).

TABLE 1. Final total protein and carbohydrate concentrations of *E. coli* B23 in streptomycin and kanamycin treatments and untreated after one hour incubation in M9 minimal media with 0.2% glycerol grown at 37ºC. Concentration values were normalized to relative cellular concentrations (Table 2)

	Control	Streptomycin	Kanamycin
Total protein concentration (μg/1x10^8 cells)	21	15	9
Total carbohydrate concentration (μg/1x10^8 cells)	10	25	23

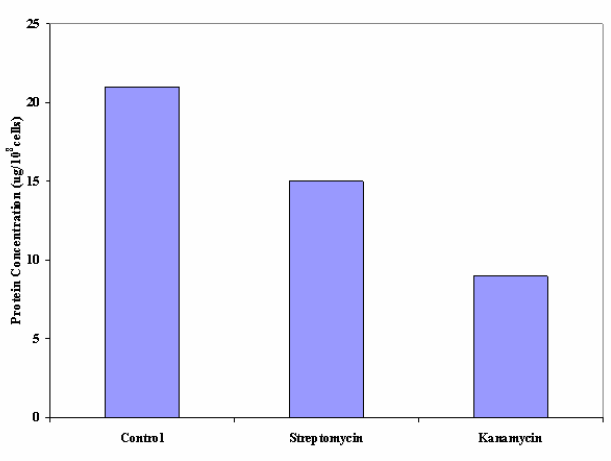

FIG 1. Total protein concentration of *E.coli* B23 cells in different conditions. "Control" refers to the absence of aminoglycosides, "Streptomycin" refers to treatment with 20 ug/ml of streptomycin and "Kanamycin" refers to treatment with 20 ug/ml of kanamycin. Cells were grown in M9 minimal media with 0.2% glycerol for 1 hour at 37°C. Cells were fractionated prior to finding total protein concentration as determined by the Bradford assay values.

Total carbohydrate concentration. The overall amount of carbohydrates increased 2.5 fold in streptomycin and 2.3 fold in kanamycin treated samples compared with the control sample (Fig 2 and Table 1).

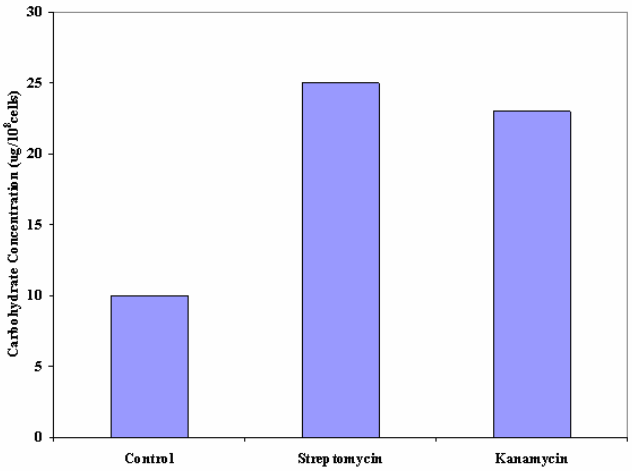

FIG 2. Total carbohydrate concentration of *E. coli* B23 cells in different conditions. "Control" refers to an absence of aminoglycosides, "Streptomycin" refers to treatment with 20 μg/ml of streptomycin during growth. "Kanamycin" refers to treatment with 20 μg/ml of kanamycin during growth. Cells were grown in M9 minimal media with 0.2% glycerol for 1 hour at 37°C. Cells were fractionated prior to measuring carbohydrate concentration as determined by the anthrone assay.

***E.coli* B23 Growth. Concentration of hexose.** The levels of hexose increased 2.6 fold for streptomycin and 3.8 fold for kanamycin treated samples when compared to control samples (Fig. 3). A concentration standard for the assay was not done due to time constraints, thus absorbance readings could not be correlated to microgram quantities.

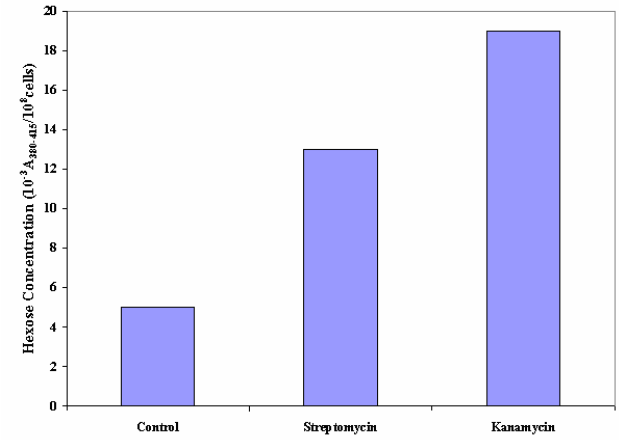

FIG 3. Hexose concentration of *E. coli* B23 cells in different conditions. "Control" refers to an absence of aminoglycosides, "Streptomycin" refers to treatment with 20 µg/ml of streptomycin during growth. "Kanamycin" refers to treatment with 20 µg/ml of kanamycin during growth. Cells were grown in M9 minimal media with 0.2% glycerol for 1 hour at 37°C. Cells were fractionated prior measuring 6-deoxyhexose concentration as determined by the hexose assay.

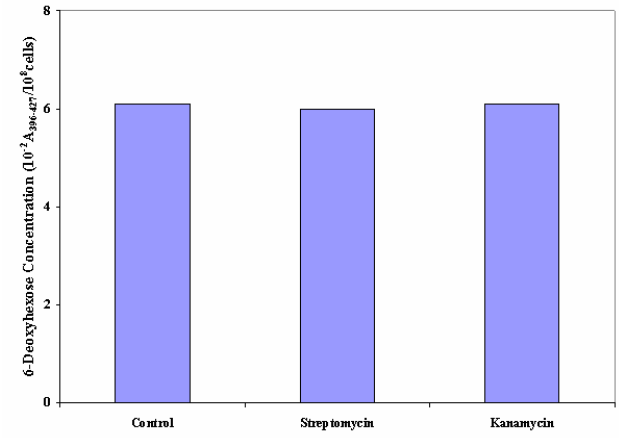

FIG 4. 6-deoxyhexose concentration of *E. coli* B23 cells in different conditions. "Control" refers to an absence of aminoglycosides, "Streptomycin" refers to treatment with 20 µg/ml of streptomycin during growth. "Kanamycin" refers to treatment with 20 µg/ml of kanamycin during growth. Cells were grown in M9 minimal media with 0.2% glycerol for 1 hour at 37°C. Cells were fractionated prior to measuring carbohydrate concentration as determined by the 6-deoxyhexose assay. Absorbance levels were normalized to units per cell by the relative growth of the samples (Table 2)

Concentration of 6-deoxyhexose. The levels of 6-deoxyhexose in the control were the same as the streptomycin sample and the kanamycin sample (Fig. 4). A concentration standard for the assay was not done due to time constraints, thus absorbance readings could not be correlated to microgram quantities.

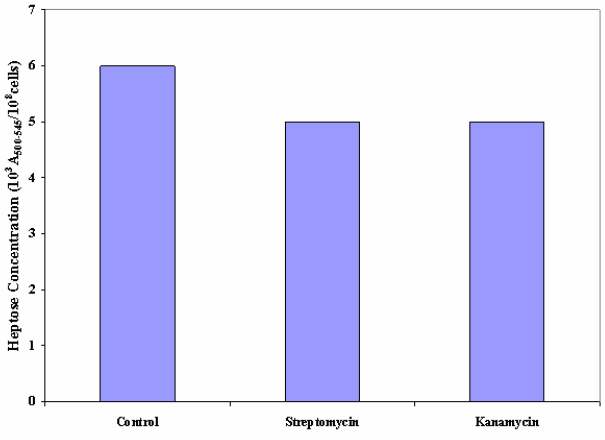

FIG 5. Heptose concentration of *E. coli* B23 cells in different conditions. "Control" refers to an absence of aminoglycosides, "Streptomycin" refers to treatment with 20 µg/ml of streptomycin during growth. "Kanamycin" refers to treatment with 20 µg/ml of kanamycin during growth. Cells were grown in M9 minimal media with 0.2% glycerol for 1 hour at 37°C. Cells were fractionated prior to measuring heptose concentration as determined by the heptose assay.

Concentration of heptose. The levels of heptose were 20% higher in the control compared to the streptomycin and kanamycin treated samples (Fig 5). A concentration standard for the assay was not done due to time constraints thus absorbance readings could not be correlated to microgram quantities.

Presence of Capsule. The results of the India ink stain showed a slightly larger capsule around the streptomycin and kanamycin treated *E.coli* B23 samples than the control
sample (data not shown).

DISCUSSION

The initial results confirmed our original hypothesis that carbohydrate production would increase in cells growing in the presence of aminoglycoside antibiotic inhibitors (Table 1 and Fig. 2). Since aminoglycosides are known to inhibit protein synthesis (2), the decrease in protein production Fig. 1 is justified. In streptomycin treated cells, there was only 75% of the amount of protein observed in control samples and in kanamycin treated samples, there was only a half of the amount of protein observed in control samples (Fig. 1). Therefore, the increase in the protein/carbohydrate

previously seen in (4) was not due to an increase in protein concentration in the antibiotic treated samples.

When observing total carbohydrate concentration in cell samples, the carbohydrate concentration increased in aminoglycoside treated cells. In streptomycin treated cells, there was approximately a 2.5-fold increase in total carbohydrate concentration whereas there was a 2.3-fold increase in kanamycin treated cells (Table 1 and Fig. 2). It appears that the increase previously observed (4) in the protein/carbohydrate in was due to total carbohydrate concentration.

In order to observe which carbohydrates were increased in order to suggest a possible explanation for this secondary effect of aminoglycosides, specific carbohydrate assays and an India ink stain were performed. The initial hypothesis was that the increase in carbohydrate was due to an increase in capsular carbohydrates, since in other studies (11), capsule size was seen to increase following antibiotic treatment. The readings of the following assays were compared relative to each other instead of relating to an absolute concentration due to the lack of a standard. However, this should be sufficient as our main goal is in the comparison of the antibiotic treatment samples to the control.

According the hexose assay, which measures mainly glucose, there was approximately a 2.6-fold increase in streptomycin treated samples and a 3.8-fold increase in kanamycin treated samples (Fig. 3). This is consistent with results seen in the Anthrone Assay (Fig. 2) in that the carbohydrate concentrations increased in antibiotic treated samples. Although this is the case, there is a discrepancy between the amounts of increase in each of the antibiotic treated samples. In the anthrone assay, the increase was greater in streptomycin treated cells, whereas in the hexose assay, the increase was greater in kanamycin treated samples. These differences may be accounted for by slight differences in the sensitivities of the assays or the methods themselves.

The 6-deoxyhexose assay theoretically assays carbohydrates associated with portions of LPS in the outer core only (5). When samples were assayed for 6-deoxyhexose, the difference between the samples per cell were virtually non-existent. (Fig 4). Therefore, this is indicative that LPS levels do not increase in the aminoglycoside treated cells.

The heptose assay measures carbohydrates associated with the inner core of LPS (5). The highest levels were seen in the control samples, which had only a 20% increase over both the antibiotic treated samples (Fig. 5). This further supports the idea that LPS levels are decreasing in the aminoglycoside treated cells.

An India ink stain technique was used to qualitatively compare the capsule composition between the control and aminoglycoside treated cells. It was seen that there was a slightly larger brown-black color around the treated cells than the control under 100X oil immersion. This indicated that there were larger capsules on treated cells than the control. It would have been better if a higher magnification was present to look at a specific cell from each condition in order to quantify the approximate differences between the overall size of the capsules.

According to literature, (10), there are more than 80 different polysaccharide K antigens, and 167 different serotypes of O antigen. Only a few different types of polysaccharides were tested in our experiment. More can be tested to definitively give an answer to what components of capsular material increased in the presence of aminoglycoside inhibition. However, as only hexose sugar levels increased significantly in the experiment, it is likely that this target component is one of the many hexose sugars.

Capsules and exopolysaccarides are known to slow down the penetration and reaction of aminoglycosides on bacteria such as *E.coli* (9). The positive charge on aminoglycosides causes it to be partially retarded by the oppositely charged nature of some components of capsular components (9). The presence of capsules, and more importantly the thickness of the layer of capsules produced, reduces the rate of diffusion since there will be more material to pass through (9). According to these previous points, capsular production of *E. coli* may protect it from low level exposure to aminoglycosides and allow it to grow, regardless of the bacteriostatic effects of the antibiotics(9). Increased transcription levels of capsule production genes may suggest that there is increased polymerization and membrane transport of capsules by *E.coli* (10). This will allow *E.coli* to avoid the complete bacteriostatic affects of these antibiotics and allow it to continue to grow (9).

Relating these above facts to our data, there are corresponding results in growth rate data. The control samples grew faster than the aminoglycoside treated samples, but the treated samples were not inhibited to a point of almost no growth. Instead the treated samples were only inhibited by about 25%, indicating that the antibiotics were at sub-inhibitory levels but enough to place a selective pressure on the samples.

Overall, it can be shown that protein synthesis is inhibited by the presence of aminoglycosides, yet the secondary effects of these antibiotics seem to promote the production of total carbohydrates of *E.coli* B23. Capsule production could very well be the cause of the increased levels of carbohydrates within the inhibited bacteria, since capsules slow down aminoglycoside penetration and therefore growth can continue.

FUTURE EXPERIMENTS

Confirming the trend that carbohydrate levels increase with aminoglycoside treatment, using different carbohydrate assays would be useful. Further refinement can be done by assaying levels of specific types of carbohydrates, such as pentoses and riboses. Comparison between cytosolic carbohydrate levels and extracellular (capsular) carbohydrate levels by fractionation of both phases would serve to support or disprove our hypothesis of increased capsule growth under aminoglycoside treatment. These phases could be separated by means of density gradient centrifugation. To determine whether capsules are the cause of this increase in carbohydrate production, investigations into the expression mechanisms of capsule production should be preformed.

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REFERENCES

1. **Bradford, M.M.** 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal. Biochem. **72**: 248-254.

2. **Davis, B.D.** 1987. Mechanism of bactericidal action of aminoglycosides. Microbiol. Rev. **51:**341-350.

3. **Gerhardt, P.** 1981. Total Carbohydrates by Anthrone Reaction. *In* P. Gerhardt (ed.), Manual of methods for general bacteriology, American Society of Microbiology, Washington, D.C.

4. **Kestell, D., S. Lai, G. Liang, S. Waters, and A. Wladichuk.** 2002. Effects of kanamycin and streptomycin on the macromolecular composition of streptomycin-sensitive and resistant *Escherichia coli* strains. J. Exp. Microbiol. Immunol. **2:**177-181.

5. **Hancock, R. E. W.** 19 September 1999, posting date. Hancock Laboratory Methods: Carbohydrate Assays. Department of Microbiology and Immunology, University of British Columbia, British Columbia, Canada. [Online] http://www.cmdr.ubc.ca/bobh/methods/CARBOHYDRATEASSAYS.html

6. **Nichols, W. W., S. M. Dorrington, M. P. E. Slack, and H. L. Walmsley.** 1988. inhibition of tobramycin diffusion by binding to alginate. Antimicrob. Agents Chemother. **32:** 518-523.

7. **Park, J. T., and R. Hancock.** 1960. A fractionation procedure for studies of the synthesis of cell-wall mucopeptide and of other polymers in cells of *Staphylococcus aureus.* J. Gen. Microbiol. **22:** 249-258.

8. **Schroeder, R., C. Waldsich, and H. Wank.** 2000. Modulation of RNA function by aminoglycoside antibiotics. EMBO. **19:** 1-9.

9. **Slack, M. P. E., and W. W. Nichols**. 1982. Antibiotic penetration through bacterial capsules and exopolysaccarides. J. Antimicrob. Chemother. **10**: 368-372.

10.**Whitfield C., and I.S. Roberts**. 1999. Structure, assembly and regulation of expression of capsules in *Escherichia coli*. Mol. Microbiol. **31**: 1307-1319.

11. **Wilson, J. W., M. J. Schurr, C. L. LeBlanc, R. Ramamurthy, K. L. Buchanan, and C. A. Nickerson.** 2002. Mechanism of bacterial pathogenicity. Postgrad Med. J. **78:** 216-224.