Journal of Experimental Microbiology and Immunology (JEMI) Vol. 13:41-46

The Effect of Aberrant Protein Synthesis on the Production of Capsular Polysaccharide in *Escherichia coli* B23

Andrew L. Wong, Daniel Y. Fong, Guilaine K. Boyce and Minhee R. Kim
Department of Microbiology & Immunology, UBC

Streptomycin, an aminoglycoside, binds to and modifies prokaryotic ribosomes leading to a decrease in protein synthesis and the production of mistranslated proteins. Previous studies reported that *Escherichia coli* B23, under sub-lethal concentrations of streptomycin, exhibited an increase in capsule polysaccharide (CPS). The mechanism through which this increase in CPS occurs was unknown. In this investigation, we hypothesized that the increase in CPS in *Escherichia coli* B23 correlated with the increase in mistranslated protein and not simply due to a decrease in protein synthesis. Mistranslation in protein was measured by changes in the Michaelis constant (K_M) and maximum reaction rate (V_{max}) of induced β-galactosidase while CPS production and protein synthesis was measured using an anthrone assay and optical density respectively. Our results are inconclusive on the correlation between protein mistranslation and CPS production but indicate that decreased protein synthesis does not consistently correlate with an increase in CPS.

Aminoglycosides are inhibitors of prokaryotic protein synthesis and are known to exhibit a pleiotropic set of effects on bacteria such as ribosomal blockage, mistranslation of protein, and membrane damage (3). Taken together, these effects are thought to contribute to the bactericidal activity of this class of antibiotic (3). Mistranslation of proteins is caused by the aminoglycoside binding to and subsequently modifying the function of the 30S ribosomal subunits of post-initiation ribosomes such that current chain elongation continues albeit with misreading. Ultimately, this results in an increase in the production of abnormal proteins (10).

A secondary effect of aminoglycosides has been observed as elevated levels of capsular polysaccharide (CPS) in *Escherichia coli* B23 in response to exposure to sub-inhibitory concentrations of aminoglycosides (2, 7). These capsular polysaccharides are composed of high molecular weight acid polysaccharides. They play a protective role by mediating the interactions of the cell with its environment and are well-known virulence factors involved in the inhibition of opsonophagocytosis during infection (18). These structures are also of great clinical significance as they are implicated in biofilm formation which confers resistance to antibiotic treatment and contribute to bacterial persistence and chronic infection (7, 18).

External stresses such as elevated temperature can cause aggregation or misfolding of proteins in the cells. These abnormal polypeptides induce a transient elevation in the levels of heat shock proteins, but also increase capsular polysaccharide synthesis by promotion of *cps* gene products (12). This thickening of the capsule is thought to be a mechanism to strengthen the cell envelope to resist environmental duress (12). The question remains, however, as to how sub-inhibitory concentrations of aminoglycosides contribute to an increase of capsular polysaccharide synthesis. We proposed testing whether this change in capsular phenotype was due to the streptomycin-induced production of aberrant proteins which have been noted to signal the upregulation of capsular polysaccharide synthesis or a general decrease in cellular protein content caused by the protein synthesis inhibition effect of the antibiotic.

We hypothesized that the presence of streptomycin-induced mistranslated proteins would correlate to an increase in capsular polysaccharide synthesis. To investigate this hypothesis, *E. coli* B23 cells were subjected to treatment with sub-lethal concentrations of two different translation-inhibiting antibiotics; streptomycin and chloramphenicol. Chloramphenicol has a bacteriostatic effect and inhibits protein synthesis by binding to a single site on the *E. coli* 50S ribosomal subunit to block peptide bond formation by the ribosomal peptidyl transferase (15, 17). Furthermore, this antibiotic is thought to significantly slow or halt protein synthesis without increasing the frequency of mistranslation allowing a comparison of the two tested variables (15).

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.

Growth of *E. coli*. An overnight culture of *E. coli* B23 was prepared by inoculating 60 mL of M9 minimal medium (8.4 mM NaCl, 48 mM Na_2HPO_4, 22 mM KH_2PO_4, 18 mM NH_4Cl, 0.80 mM $MgSO_4.7H_20$ and 0.4% (w/v) glycerol) with a loopful of an isolated colony of *E. coli* B23. The inoculated culture was placed overnight in a shaking incubator (G10 Gyrotory Shaker) at 37°C at 200 rpm.

Antibiotic stock solution. Stock solutions of 20 mg/mL of streptomycin sulphate (IGN-#100556) and 5 mg/mL of chloramphenicol (Sigma-Aldrich, #C-1919) were prepared by dissolving streptomycin sulphate and chloramphenicol in distilled water and absolute ethanol respectively. The resulting solution was then filter sterilized using a Millipore 0.2 µm GSWP nitrocellulose membrane. The sterilized antibiotic solutions were stored at -20°C.

Antibiotic sensitivity assay-determination of minimum inhibitory concentration (MIC). Concentration standards were prepared in 18 x 120 mm glass test tubes for streptomycin (0 µg/mL - 20 µg/mL) and chloramphenicol (0 µg/mL - 12 µg/mL) using the respective antibiotic stock solutions and M9 minimal media as the diluent to give a total volume of 4 mL of solution in each tube. An inoculum of 0.1 mL of overnight *E. coli* B23 culture was added to each antibiotic standard. The test tubes were carefully vortexed and loaded onto a shaking incubator, and incubated overnight at 37°C and 200 rpm. The turbidity of the MIC assay tubes samples were read at a wavelength of 460 nm using the Spectronic 20D spectrophotometer.

Subinhibitory antibiotic treatment. Three growth treatments; control, streptomycin and chloramphenicol, were prepared in three separate 1 L Erlenmeyer flasks each containing 187 mL of M9 minimal media and 13 mL of the overnight *E. coli* B23 bacterial culture. Stock solution streptomycin was added to a final concentration of 4 µg/mL to the streptomycin –treated culture and stock solution chloramphenicol was added to a final concentration of 6 µg/mL to the chloramphenicol-treated culture. The third flask served as the untreated control. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (BRL, #5529UA) was added to a final concentration of 0.5 mM to each flask. The three flasks were then placed in a shaking incubator at 37°C and 200 rpm for a 5 hour time period. Subinhibitory concentrations of antibiotic were determined via MIC assays.

Standardization of concentration of cultures after antibiotic treatment. At the end of the 5 hour incubation period, the turbidity of the two treatment cultures and the control was measured at wavelength 460 nm using the Spectronic 20 spectrophotometer blanked with 3 mL of M9 minimal medium. In order for the three cultures to have approximately the same amount of culture mass, the two cultures with the higher OD_{460nm} readings were diluted to equal the OD_{460nm} of the lowest culture.

Isolation of intracellular protein and enzyme extraction. 100 mL of each treated culture and the control was transferred from the flasks and centrifuged in a Beckman J-21 high-speed centrifuge at 7500 x g for 10 min. The supernatant was then discarded and the remaining cell pellets were resuspended in 0.9 mL of TM buffer (Tris (pH 8.0, 10 mM), $MgCl_2$ (0.1 mM)) and chilled on ice for 3 min. The samples were then transferred into three separate Fisherbrand 2 ml screw-cap microfuge tubes (Fisher, #05-664-34), to which 0.1 mL of 0.1 mm glass beads (BioSpec Products Inc., #11079107) were added. The tubes were placed in the Fast Prep Cell Disrupter (Bio 101, FP120A-115) and agitated for three consecutive 30 s bursts at power setting 6 with placement of the tubes on ice for one minute between bursts. Once breakage of cells was completed, the samples were incubated at 30°C with 0.05 mL of 300 µg/mL DNase (Sigma-Aldrich, #D-5025) and of 300 µg/mL RNase (Sigma-Aldrich, #R-5125) for a 15 min. After the nucleic acid digestion, the tubes containing the samples were chilled on ice for at least 4 min and then centrifuged in microcentrifuge (Eppendorf centrifuge 5415D) at 14 000 x g for 5 min. To remove residual particulate matter, the samples were filtered using a 0.45µm pore-size particle filter (Millipore®, #RINN33660). TM buffer was added to the filtered broken cell supernatants if necessary to obtain a final total volume of 0.9 mL.

Bradford Assay. A standardization step was performed earlier such that the protein concentration of each treatment would be approximately the same. In order to verify this assumption, a Bradford assay was performed to measure total protein concentration in the three supernatants from the two treatment cultures and the control culture. Bradford standards ranging from 0 µg/mL to 80 µg/mL of chicken egg albumin (Sigma-Aldrich, #A-5253) were prepared by combining with 2 mL of Bradford dye reagent, incubated for 10 minutes and then read at wavelength 595 nm using the Spectronic 20 spectrophotometer. The samples of FBSC from the control and each antibiotic treatment were diluted with TM buffer to a final volume of 1 mL and then treated in an identical manner to the standards.

ONPG β-galactosidase enzyme assay. The enzyme, β-galactosidase, from *E. coli* was used as the model intracellular protein of *E. coli* with the assumption that β-galactosidase would be produced as a constant fraction of total protein content in the two treated cultures and the control. To measure aberrancy, the assumption was made that mistranslation of β-galactosidase would lead to an abnormal folding of the enzyme's active site and a resulting decrease in substrate binding efficiency, which was measured by changes in the Michaelis constant (K_M), and in catalytic efficiency, which was measured by changes in the maximum reaction rate (V_{max}). The following volumes of 5 mM ONPG (Sigma-Aldrich, #N-1127)-0.5 mL, 0.4 mL,0.3 mL, 0.2 mL, 0.1 mL, 0.0 mL-were each placed into a 18 x 120 mm test tube and TM buffer was added to give a total final volume of 3.6 mL. A volume of 0.1 mL of the enzyme extract for the control treatment was added to a test tube of ONPG and TM buffer and the contents of the test tube were immediately vortexed and then transferred to the cuvette and the absorbance at wavelength 420 nm was recorded for 5 minutes or until an absorbance of 1.0 was reached. This was carried out for each volume of ONPG. This ONPG assay was repeated using 0.015 mL of chloramphenicol-treated FBCS per test tube and 0.05 mL of streptomycin-treated FBCS per test tube.

Isolation of capsular polysaccharide. Isolation of the capsular polysaccharide was performed with some modifications to the protocol outlined by Ganal *et al*. (5). A volume of 100 mL of each bacterial culture was centrifuged using a Beckman J2-21 centrifuge 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 1.4 mM Na_2HPO_4, 1.8 mM KH_2PO_4) to ¼ of the original volume. PBS resuspended pellets were blended in an Osterizer Cycle Blend Waring blender for 5 min set at "Blend". Exopolymers were then precipitated by adding two volumes of ice-cold acetone. The resulting suspension was centrifuged using a Beckman J2-21 centrifuge 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 stored at -4°C.

Purification of capsular polysaccharide. The crude exopolymer extracts were placed in Spectra/Por® molecularporus membrane dialysis tubes with a molecular weight cut-off of 6,000-800 kDa. These samples were dialyzed against 1 L of distilled water at 4°C for 24 h. The dialyzed exopolymers were dried overnight using a lyophilizer (Alcatel, 3110). The dried crude exopolymers were dissolved in 20 mL of sterile 10 mM $MgCl_2$. Deoxyribonuclease I (DNase I) (Sigma-Aldrich, #2326670) and ribonuclease A (RNase A) (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 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 at 16,000 x g for 5 min. Purified capsule contained in the supernatants was stored at 4°C until use.

Anthrone carbohydrate assay. Anthrone reagent was prepared by dissolving 100 mg of anthrone (MC&B-#AX1655) in 2.5 mL of absolute ethanol. The solution was then made up to 50 mL using concentrated (75%) sulfuric acid and chilled on ice until used (1). Glucose standards ranging from 0 mg/mL to 100 µg/mL were prepared using α-D(+) glucose (Sigma-Aldrich, #G-5000) and the capsular polysaccharide resuspension solution (0.05 M Tris-base, 0.1 M NaCl) as diluent to give a total final volume of 0.6 mL. An aliquot of 2.5 mL of chilled anthrone reagent was added to each glucose standard and vortexed. The tubes were then incubated in a stationary 100°C water bath for 10 min after which they were transferred to an ice bath until cooled. The absorbance at wavelength 625nm was then read for each glucose standard using the Spectronic 20D spectrophotometer. The experimental samples of purified capsular polysaccharide were treated in an identical method to measure the concentration of carbohydrate present.

RESULTS

Intracellular protein concentration. Protein synthesis, as reflected by culture turbidity before partial protein purification, was found to be highest in cells grown within the control condition (Table 1). The optical density readings showed a 55-60% decrease in the amount of protein synthesis in antibiotic treated cells. Bradford assay data allowed confirmation of standardization between samples but the concentration of total partially purified protein was approximately 1.3 times higher in the control condition than in cells incubated with chloramphenicol and approximately 1.2 times higher than in cells incubated with streptomycin (Table 1). As the exact difference between the streptomycin and chloramphenicol treatments is only 9.5%, it brings to question if the results display a significant difference or not. This concept, coupled with a lack of replicates, indicates that there may not be a difference in the concentration of total partially purified protein. In fact, mean absolute deviations show that measured intracellular protein concentration in all treatment conditions overlap. This lack of a difference is anticipated as culture turbidities were standardized in the experiment and is assumed to be correlated with intracellular protein concentrations.

Production capsular polysaccharide (CPS). Total concentration of CPS from cells harvested from each 5 hour treatment condition was measured (Table 1). The observed levels of polysaccharide from cells of the streptomycin treatment differed significantly from those of chloramphenicol treatment and from those of the control (inferred using mean absolute deviation values). However, no significant difference in levels of polysaccharide was found between cells from the chloramphenicol treatment and the control. The concentration of CPS is highest in the cells treated with streptomycin, as expected. This is 16% higher than the concentration of CPS in the cells under the control condition and 22% higher than the concentration of cells treated with chloramphenicol. The concentration of CPS for cells in the control condition is solely 5% higher than the CPS concentration of cells incubated with chloramphenicol, which may indicate that there is no significance difference in CPS levels between the control condition and cells incubated in chloramphenicol as was expected.

DISCUSSION

The results indicate that the presence of protein mistranslation, as measured by an increase in the K_M and a decrease in V_{max} of β-galactosidase, does not correlate with an increase in CPS. *E. coli* B23 cells treated with chloramphenicol or with streptomycin both resulted in an increase of K_M and a decrease in V_{max} in β-galactosidase compared to the control, but these two treatments differed with an increase in CPS for cells treated with streptomycin and a decrease in CPS for cells treated with chloramphenicol (Table 1). Chloramphenicol is known to block the initiation step

TABLE 1. Maximum theoretical reaction rate and substrate binding efficiency of β-galactosidase, intracellular protein concentration, and level of capsular polysaccharide isolated from *E. coli* B23[a].

Treatment	V_{max} (units/mL)	K_M (mM ONPG)	Initial turbidity (OD_{460nm} units)	partially purified protein [mg/mL]	Capsular polysaccharide [mg/mL]
Control	5.05 ± 0.204	0.27 ± 0.036	0.275	2.7 ± 1.06	23.3 ± 1.52
Chloramphenicol	2.62 ± 0.256	0.41 ± 0.071	0.177	2.1 ± 1.06	22.2 ± 1.52
Streptomycin	1.64 ± 0.309	0.37 ± 0.128	0.170	2.3 ± 1.06	27.0 ± 1.52

Data represents 1.7×10^{11} cells

in protein synthesis leading to an absence of protein synthesis (11, 19). If protein translation should still occur, it should be completed with regular fidelity. Taken together, this suggests that the changes in K_M and V_{max} are not related to chloramphenicol inhibition.

Sypherd *et al.* (15) and Horiuchi *et al.* (8) suggest the incubation with chloramphenicol should not induce mistranslation as they reported no detectable difference in K_M between cell-free extracts of chloramphenicol treated cells and their untreated control, a result that differed from our observed 50% increase in K_M of β-galactosidase incubated with chloramphenicol versus the untreated control. However, there was also evidence that chloramphenicol may lead to the formation of incomplete transcripts of β-galactosidase which would theoretically translate into incomplete enzyme (11). These incomplete enzymes could possibly cleave the ONPG substrate but have a decreased ability to bind substrate and to catalyze the formation of orthno-nitrophenol as measured by K_M and V_{max} respectively; a speculation supported by our observation of an increased K_M and decreased V_{max} of β-galactosidase in *E. coli* treated with chloramphenicol. These conflicting results suggest that the question still remains open whether the presence of mistranslation relates to an increase in CPS if chloramphenicol causes mistranslation.

Streptomycin, on the other hand, is known to affect protein fidelity and this is reflected in our results through a decrease in V_{max} of β-galactosidase. This observation has been documented in the past by Goldemberg and Algranati (6) who measured mistranslation via a leucine and phenylalanine incorporation assay on poly(U) transcripts and by Pinkett *et al.* (14) who observed that there was a cessation in β-galactosidase activity despite continual growth of cells, a possible indicator that cells treated with streptomycin had ceased to produce functional β-galactosidase (6, 14). Streptomycin is known to interact with the "A" site and the "P" site of bacterial ribosomes which leads to the production of aberrant protein as a result of protein mistranslation and early termination due to disruption of the ribosomal cycle (14). This decrease in protein fidelity, measured via a decrease in V_{max} was observed alongside an increase in CPS which would suggest that protein fidelity may be correlated to the production of CPS. However, as the K_M of β-galactosidase produced under treatment with streptomycin was not demonstrably different from that of the chloramphenicol condition or untreated conditions (Table 1), our results have refuted the idea that mistranslated protein correlates with increases in CPS production.

In addition to protein mistranslation, another major outcome of aminoglycosides is a decrease in protein synthesis, which was measured through optical density. However, our results indicated that this decrease in protein synthesis did not correlate with an increase in capsule polysaccharide synthesis. Specifically, we observed that incubating *E. coli* with chloramphenicol or streptomycin decreased protein synthesis when compared to a control, but that CPS increased only in cells treated with streptomycin and decreased in *E. coli* treated with chloramphenicol. The correlation between reduced protein synthesis and reduced CPS in the chloramphenicol treatment corresponded to work by Whitfield (18) who demonstrated that protein synthesis is required for CPS production via the usage of chloramphenicol in temperature up-shift experiments.

The correlation of reduced CPS with reduced protein synthesis drawn from the chloramphenicol treatment was in direct conflict with the observed increase of CPS production alongside a decrease in protein synthesis for cells incubated with streptomycin (Table 1), a result that has been reported in the literature (2). A possible explanation for the observed result occurring in streptomycin but not chloramphenicol lies in the work of Pinkett *et al.* (14). Pinkett *et al.* observed that streptomycin requires 5-10 minutes in order to have an active effect on the cells. Although our treatment incubation occurred over 5 hours to compensate for this, we used a lower concentration of streptomycin compared to Pinkett *et al.* and this lag time may have been increased within our experiment (14). This increased lag time would allow the production of more mistranslated yet partially dysfunctional enzyme to produce the observed increase in CPS. The loss of these aberrant proteins would then subsequently occur, as the work of Dukan *et al.* (4) indicates that streptomycin mistranslation leads to a carbonylation of proteins. Dukan *et al.* (4) observed that this carbonylation causes a two-fold increase in susceptibility towards enzymatic degradation within the cell. Taken together, this two-part mechanism based from the work of Pinkett *et al.* (14) and Dukan *et al.* (4) could provide an explanation for the observed phenomena, but this is relatively unlikely as the mistranslated protein would maintain lowered functionality and would also be degraded before it has a significant effect on CPS production (4, 14).

Altogether, our results indicated that the inhibition of protein synthesis caused by streptomycin did not explain the observed upregulation of CPS as the inhibition of protein synthesis by chloramphenicol did not produce the same effect. Furthermore, we were unable to conclusively determine if the production of aberrant or mistranslation protein caused by streptomycin's effects on ribosomes correlated to an upregulation of CPS. This inability to support the hypothesis stemmed from the unexpected high K_M and low V_{max} of the model enzyme in chloramphenicol incubated cells, which would indicate a high level of

aberrancy. Another contributing factor to this was the associated error in the K_M of β-galactosidase which was not demonstrably different from the K_M of β-galactosidase from the chloramphenicol culture and untreated culture. As the putatively aberrant proteins in chloramphenicol did not produce an upregulation of CPS, our results implicate that an increase in protein mistranslation does not correlate to an increase in CPS production.

FUTURE EXPERIMENTS

One of the limitations encountered in this experiment was that the Michaelis constant and maximum reaction rate were the only measure of mistranslated proteins and it was assumed that enzyme activity of β-galactosidase would serve as an accurate representation of mistranslation of the enzyme. Alternative assays would help to accurately ascertain the presence of mistranslated protein in the cells. Examples of such assays would include an assay used by Goldemberg and Algranati (6) where the ratio of leucine to phenylalanine incorporation into polypeptides was measured in the presence of poly(U) as mRNA (6). Another technique would be the use of isoelectric focusing. In mistranslation, the substituted amino acid may differ in charge from the normal amino acid leading to a difference in the charge from the normal protein and this can be separated and quantitated using this technique (13). Finally, prior literature indicates that chloramphenicol may specifically inhibit β-galactosidase as well as decreasing general protein synthesis (15); therefore another model protein should be selected for future studies.

Another side effect of aminoglycosides is the mimicry of a starvation signal. This could lead to the triggering of a stringent response in affected cells which may explain the upregulation of capsular polysaccharide in cells incubated with subinhibitory levels of aminoglycosides. As the alarmone, guanosine tetraphosphate (ppGpp), is the primary signal of the stringent response; it may be useful to test for the presence of this molecule the cells treated with aminoglycosides or to test the levels of CPS in strains that have deletions in *relA* and *spoT* (genes encoding for ppGpp synthetases) (16).

The use of other mutants might provide insight into these possible mechanisms by which capsule synthesis is upregulated. For example, the effects of streptomycin-induced mistranslation on the Lon protease, a negative regulator of the capsule polysaccharide synthesis (*cps*) operon, may be involved in this observed secondary effect of aminoglycosides. Use of streptomycin-sensitive and streptomycin-resistant *lon* capable (wild type) cells, as well as cells with reduced *lon* activity (or *RcsA* with reduced binding to Lon but not RcsB), along with appropriate controls may elucidate on the involvement of *lon* protease. A streptomycin resistant cell should not show this phenotype. Partially defective *lon* mutants will expectedly show an increased capsule but whether or not it shows an increase in this mucoid phenotype under streptomycin treatment may indicate possible involvement of these molecules in response to this antbiotic stress.

Other strains that might prove helpful as controls include *rpsD* (coding for the 30S ribosomal protein S4) mutants which, similar to streptomycin, exhibits ribosomal ambiguity which is characterized by lowered discrimination between cognate and non-cognate tRNAs (9).

ACKNOWLEDGEMENTS

We would like to extend our sincerest gratitude to Dr. William Ramey for providing his time and laboratory supplies throughout our investigation, and Shaan Gellatly for her guidance. As well, we would like to thank the media room staff for providing us with autoclaved materials. In particular, we greatly appreciate the efforts of Lando Robillo and Nick Cheng for accommodating us with the lyophilizer and providing a second opinion for key aspects of our project. Lastly, we acknowledge the efforts of our fellow peers and colleagues in the Department of Microbiology and Immunology, UBC for the sharing of equipment and of stock solutions.

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