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Osmotic Stress Induces Kanamycin Resistance in *Escherichia coli* B23 through Increased Capsule Formation

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In order to test whether capsule that is induced by non-antibiotic stress can render antibiotic resistance to microbes, this study explored the changes in capsule formation in response to sodium chloride osmotic pressure in *Escherichia coli* B23 and its protective effects against the bacteriostatic activity of kanamycin. Capsules were induced by incubating cells in four different NaCl concentrations, 0.085 M (control), 0.2 M, 0.4 M and 0.6 M. Capsules were then extracted and size was examined by determining glucose and fucose concentrations using anthrone assay and 6-deoxyhexose assay, respectively. Induced cultures were plated on Luria-Bertani plates supplemented with kanamycin at various concentrations and colony forming units were then counted. Increased NaCl concentration led to greater capsule sizes, and increased survival rate when the cells were exposed to the antibiotic. These results elucidated that capsules induced by NaCl osmotic pressure had a protective effect against kanamycin and allowed for better cell survival.

Escherichia coli is a rod-shaped, Gram negative bacteria that is enclosed by a polysaccharide capsule (13). The capsule provides a protective barrier, facilitates adhesion, and determines virulence by inhibiting non-specific and specific host immune responses (13). Most *E. coli* capsules are primarily composed of lipopolysacharide associated O antigens and capsular K antigens. There is incredible variability in the structure of K and O antigens reported (8). Certain mucoid *E. coli* strains produce colanic acid (M antigen) capsule which is composed of glucose, galactose, fucose and glucoronic acid (1). Colanic acid is thought to be important for cell survival outside of the host, protecting the cell from an array of environmental stressors (20).

Capsule formation is regulated by Regulator of Capsule Synthesis (*rcs*) genes whose main regulatory proteins are RcsA, RcsB and RcsC (18). Among the three, RcsB, the constitutively expressed response regulator protein, is most essential for capsule synthesis (6). Upon activation of the regulatory network, RcsB and RcsA form a heterodimer that acts as a transcription factor. RcsA protein is an auxiliary regulatory protein that may or may not be involved in gene activation and its level is closely monitored and maintained by Lon protease (9). RcsC is identified as a sensor kinase that phosphorylates RcsB and is constitutively expressed. Rcs proteins have been shown to regulate over 150 genes including the ones involved in antibiotic resistance and capsular polysaccharides (14) and colanic acid synthesis (7). Earlier studies have shown that the Rcs regulatory network can be activated by stimuli such as exposure to antibiotic stress, osmotic pressure, envelope stress and overproduction of specific membrane components (14).

Bacterial exposure to sub-lethal levels of antibiotics has been shown to increase capsular polysaccharide formation and resistance to other unrelated antibiotics (16). A previous study investigated the effect of capsular polysaccharides on non-specific antibiotic resistance by using two mutant strains of *E. coli,* 2205-2 and JW0429-1, which over-produce and under-produce capsular polysaccharides respectively, and examining their growth when cultured in sub-lethal dose of kanamycin and streptomycin (2). No significant difference in survival rate was observed despite varying levels of capsule formation, indicating that capsule formation in response to antibiotic stress did not attribute to the observed antibiotic resistance.

Given that previous studies failed to find a link between capsule formation and resistance, we aimed to investigate the role of an osmotic effect on capsule formation and antibiotic resistance. Besides antibiotics exposure, osmotic pressure can also activate the Rcs regulatory network to induce capsule production, and thus perhaps enhance survival to non-specific antibiotic stress. In this study, bacterial capsule formation was induced using sodium chloride (NaCl) osmotic pressure. Following induction, the cells were exposed to kanamycin as an antibiotic stress to investigate whether

an osmotic pressure induced capsule can render protection against kanamycin. Ultimately, results from this study showed increased capsular polysaccharide production and kanamycin resistance with increasing NaCl osmotic pressure, indicating that high concentration of NaCl induces capsule which has protective effects against bacteriostatic activity of kanamycin.

MATERIALS AND METHODS

Cell Strain, growth condition and media. *Escherichia coli* B23 was used for the investigation of induced capsule on protection against kanamycin (Sigma, K-4000). The cells were cultured using Luria-Bertani (LB) liquid media broth (1.0% w/v tryptone and 0.5% w/v yeast extract) or plates (LB with 1.5% w/v agar) with NaCl concentrations at 0.085, 0.2, 0.4, and 0.6 M. Cultures were incubated at 37**ºC,** with liquid cultures shaken at a minimum speed of 190 rpm.

Kanamycin inhibition growth assay. Overnight starter culture was inoculated into four different NaCl concentration levels (0.085, 0.2, 0.4, and 0.6 M) for a minimum of five hours to induce capsule production. Induced cell cultures were diluted to 10^{-6} cfu/ml, and then 100 µl was plated in duplicate onto LB agar using beads. Plates had the same concentrations of NaCl as used for capsule induction and were supplemented with 0, 1.5, 2.0, 2.5, or 3.0 µg/ml kanamycin. Only plates within the 30 to 300 CFU/plate range after overnight incubation were counted and used for analysis.

Capsule extraction. Capsular extraction was carried out as described by Lu *et al.* with a few modifications (12). Sixty ml of induced cell cultures were centrifuged at 7300*g* (Beckman J2-21 Centrifuge with JA-20 Rotor) for 10 minutes. Supernatants were discarded and cell pellets were resuspended in 10 ml of PBS (137 mM NaCl, 2.7 mM KCl, 1.4 mM Na_2HPO_4, and 1.8 mM KH_2PO_4). Resuspended cells were blended for 1 minute, then 20 ml of ice chilled acetone were added to blended cells. Samples were centrifuged at 7300*g* for 10 minutes. Supernatants were discarded and pellets were resuspended in 2.0 ml PBS. Samples were dialyzed using 1 ml/cm Spectra/Por® molecularporous membrane dialysis tubes at 4°C for 24 to 48 hours using 1 to 2 L of distilled water and were then lyophilized for 24 to 48 hours. The freeze dried samples were dissolved in 3 ml of 10 mM $MgCl_2$. RNase A (Sigma, R-4875) and DNase I (Sigma, D-5025) were added to each sample at a final concentration of 0.1 mg/ml and 5 µg/ml, respectively, then samples were incubated for 3 hours at 37°C in a shaking water bath. Pronase (Boehringer Mannheim, 1459643) was then added to a final concentration of 0.1 mg/ml and samples were incubated overnight in a 37°C shaking water bath. Samples were then heated at 80°C for 30 minutes and then centrifuged for 20 minutes at 15000*g*. Supernatants were dialyzed and lyophilized as described above.

Anthrone carbohydrate assay. This assay was performed as described by Lu *et al.* with minor modifications (12). All reagents were kept on ice during the assay unless otherwise noted. Anthrone reagent (Matheson Coleman & Bell, AX1655) was prepared by dissolving 200 mg of anthrone into 5 ml of 100 % ethanol, then adjusted to 100 ml using concentrated sulfuric acid. Glucose standards (L-Glucose Anhydrous, Sigma) were prepared at concentrations of 500, 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, and 0 µg/ml by serial dilution using dH_2O. Freeze dried capsule extractions (CPS) samples were resuspended in 2.2 ml of dH_2O, then 0.5 ml of each sample duplicates or glucose standard was combined with 3 ml of anthrone reagent. After vortexing, samples and standards were incubated at 100°C for 10 minutes. After incubation, the samples and standard tubes were placed on ice to stop the reaction before reading absorbance at 625 nm. The measured glucose concentrations were then standardized for total cell numbers by dividing glucose concentrations by turbidity reading of the cells following 5 hour induction.

6-deoxyhexose assay for fucose. This assay was performed as described by Ganal *et al* (3) with minor modifications. All reagents were kept on ice during the assay unless otherwise noted. Fucose standards were prepared at concentrations of 480, 400, 300, 200, 100, 50, 25, 12.5, and 0 µg/ml using dH2O. Freeze dried CPS samples were resuspended in 2.2 ml of dH_2O, then 3 ml of Reagent A (86% H_2SO_4) was added into 0.6 ml of samples and standards (prepared in duplicate) and then boiled for 5 min. After cooling, 60 µl of freshly made Reagent B (3.0 % w/v cysteine HCl) was added to each sample or standard. Tubes were incubated for 3 hours in the dark on a shaker at room temperature. Absorbance readings were taken at 396 nm and 427 nm. The measured fucose concentrations were then standardized for total cell numbers by dividing fucose concentrations by turbidity reading of the cells following 5 hour induction.

Maneval's Capsule Staining Method. This assay was performed as described by Hughes and Smith (7) with minor modifications. A drop of liquid cell culture was mixed with a drop of Congo Red (1% aqueous solution, Sigma Chemical Company C-6767) and air-dried. Dried smears were counterstained for five minutes with two to three drops of Maneval's solution (0.0064% aqueous acid fuchsin, JT Baker Chemicals, A355-3; 0.51% aqueous ferric chloride, Fisher Scientific I-89; 0.77% aqueous glacial acetic acid, Acros, 42322-0025; 0.64% aqueous phenol solution, Invitrogen IS509-037). Excess stain was poured off. Slides were gently washed with dH_2O and air-dried. Slides were observed under 1000x magnification with immersion oil on a light microscope.

Effect of NaCl on kanamycin activity. Overnight starter cultures were inoculated into LB containing 0, 1.5, or 3.0 µg/ml of kanamycin at either 0.085, 0.2, 0.4, or 0.6 M NaCl for five hours. The cells used were not induced with NaCl osmotic pressure for capsule production. The survival rate was calculated using turbidity readings at 600 nm and converting the measurements into percentage scale as compared to the 0 µg/ml of kanamycin treatment at every NaCl concentration.

Data analysis. Graphs were constructed using average data from duplicate readings of all three experimental trials. Glucose, fucose and colony count results were normalized to turbidity of cell cultures following induction by NaCl osmotic pressure. Statistical significance was calculated using ANOVA and T-test assuming unequal variances at 95% confidence interval.

RESULTS

Anthrone and fucose assay demonstrated an increase in capsular size. The effect of osmotic pressure on capsule size was measured by the presence of capsular carbohydrates glucose (Fig. 1) and fucose (Fig. 2). When compared to the glucose concentration at 0.085 M NaCl condition, a significant increase was only observed in the cells exposed to 0.6 M NaCl, with a 1.5-fold increase over the control ($p = 0.036$). Glucose production in cells exposed to 0.2 and 0.4 M NaCl were not significantly different from the control ($p=0.96$ and $p=0.21$, respectively). However, there appeared to be an increasing trend in glucose concentration with increasing NaCl concentrations (Fig. 1). Fucose concentration also appeared to increase directly with NaCl concentration (Fig. 2). Fucose concentration

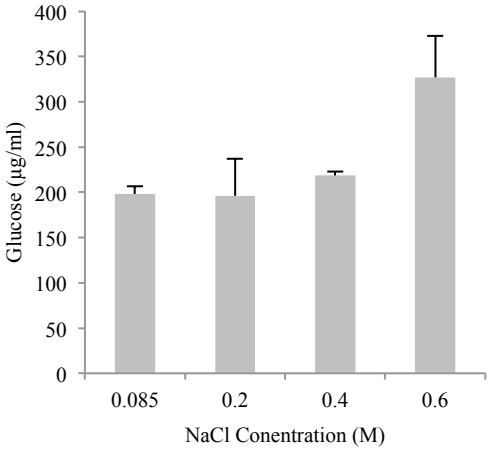

FIG. 1. Effect of osmotic pressure on capsular glucose in *E. coli* B23 cells.

increased by 2.2x, 4.2x and 4.4x the 0.085 M condition (control) for 0.2 M, 0.4 M and 0.6 M of NaCl respectively. However, difference between the different NaCl conditions were not statistically significant as determined by ANOVA analysis (p = 0.17). Overall, the increased levels of glucose and fucose indirectly suggest that capsule size increased in the *E. coli* cells subjected to higher NaCl osmotic pressure.

Maneval's stain. No images of Maneval's stained cells were included in this report as the stain was not effective and no stained capsules were observed. Cells were clumped and clustered together with stain particulates.

Percentage survival of induced cells. In order to investigate survival of osmotically induced cells in the presence of various concentrations of kanamycin, a growth assay was conducted to collect colony forming units (CFUs) on LB plates. Based on the collected CFU data, a percent survival graph was constructed (Fig. 3). As expected, with increasing kanamycin concentrations from 1.5 to 3.0 µg/ml, survival rate decreased for all NaCl conditions. At the control NaCl condition (0.085 M), cell growth was completely inhibited by 2.0 µg/ml of kanamycin. At 0.2, 0.4, and 0.6 M NaCl conditions, only partial inhibition of cell growth was observed at any kanamycin concentration (Fig. 3). In fact, the percentage survival increased directly with NaCl concentration. At 3.0 µg/ml of kanamycin, survival in the 0.6 M NaCl condition was 1.8-fold and 13-fold higher than 0.4 M and 0.2 M NaCl conditions, respectively. Similarly, it was evident that the rate of survival decline was the highest in the control, followed by 0.2 M, then 0.4 M, and lastly at 0.6 M as indicated by the slope of the line graphs in Fig. 3. The percentage survival between 0.085 and 0.2 M condition was not significant (p=0.35). Nonetheless, the difference in percentage survival was significant for 0.4 and 0.6 M NaCl conditions when compared to the control condition (p=0.046, 0.022). The data suggested that osmotic pressure-induced cells were protected at higher concentrations of kanamycin, leading to better survival.

Effect of salt on kanamycin's chemical activity. The effect of high NaCl concentrations on kanamycin was also examined to ensure that decreased kanamycin activity was not due to NaCl ionic interference at the concentrations used (Fig. 4). Cell survival was quantified using turbidity readings instead of plate counts. NaCl did not seem to have significant effect on the kanamycin function as percentage survival between the different NaCl conditions was not significant (p = 0.52).

DISCUSSION

E. coli B23 colonies have a characteristic mucoid appearance due to the presence of colanic acid (5). Colanic acid is composed of repeating units of glucose, galactose, glucuronic acid and fucose (1, 17). This high molecular weight polymer is pelleted during capsule extraction. Although most cells contain both intracellular and extracellular glucose, most of the monomeric glucose found intracellularly will stay in the supernatant, which was subsequently discarded. However, intracellular glucose in polymeric form for

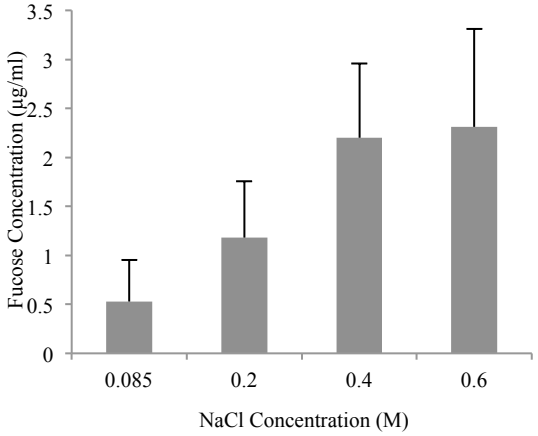

FIG. 2. Effect of osmotic pressure on capsular fucose in *E. coli* B23 cells.

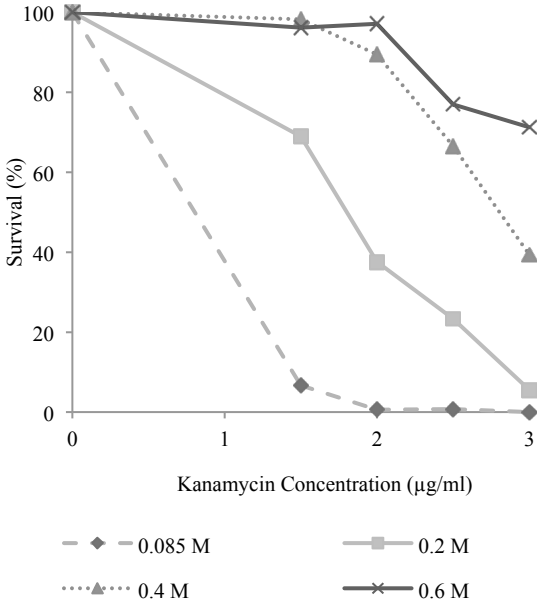

FIG. 3. Percentage survival of *E. coli* B23 cells subjected to osmotic pressure by various concentrations of NaCl in the presence of Kanamycin

energy storage could be present in the final capsule extract (4). By standardizing the glucose concentrations to cell density, it was assumed that intracellular glucose concentrations remain constant with ionic stress and did not account for the observed increase in glucose concentrations at higher NaCl concentrations (Fig. 1).

The fucose assay also showed a similar increasing trend with increasing NaCl concentrations (Fig. 2). Unlike glucose, which is common both inside and outside the cells, fucose is more commonly found extracellularly within colanic acid residues and other capsular polysaccharide molecules (19). The increasing trend of fucose production directly correlated with increasing NaCl concentration suggesting that NaCl played a role in inducing fucose production which corresponds to an increase in capsule size.

Colanic acid production is mediated by genes located in the *cps* locus (20). Specifically, *cpsB* and *cpsG* encode mannose-1-phosphate guanyltransferase and phosphomannomutase, respectively, which are responsible for the production of GDP-mannose and GDP-fucose (17). The Rcs stress-response sensory system regulates the expression of genes in the *cps* locus (20). Rcs proteins are highly conserved and are sensitive to environmental stress. Colanic acid is up-regulated in response to high osmotic pressure and for survival outside of the host (20). Thus, the concurrent increase in glucose (Fig. 1) and fucose (Fig. 2) concentrations at higher osmotic pressures indicated the induction of colanic acid production, and likely correlated to increased capsule size. These findings are consistent with previous studies where osmotic pressure was shown to cause an induction of capsule formation (14). Similar stress sensory and regulatory pathways are also seen in *Klebsiella pneumoniae, Erwinia amylovora,* and *Salmonella typhi* (20).

Kanamycin is an aminoglycoside antibiotic (21) that binds to 16S rRNA of the 30S subunit of the bacterial ribosome. It blocks protein synthesis by preventing the 50S subunit from binding and completing the formation of active ribosomes (21).The presence of capsule could confer protection against kanamycin as observed in the experimental trials of this study. Capsules may act as a physical barrier to minimize the penetration of kanamycin (15). Although physically blocking kanamycin entry may account for the observed higher survival in induced cells, a confounding factor may be that the energy-dependent translocation of kanamycin across the bacterial cytoplasmic membrane could be inhibited by hyperosmolarity (15). Kanamycin contains amino acid side groups that may become charged in aqueous condition; hence NaCl ions may interfere with the activity of kanamycin and lead to its decreased efficacy

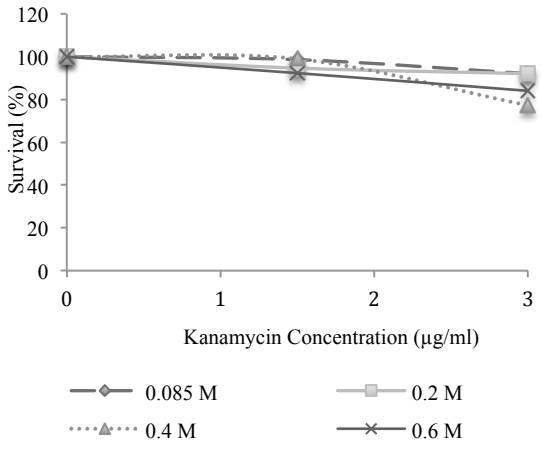

FIG. 4. Effect of NaCl on the bacteriostatic effect of kanamycin in uninduced *E. coli* B23 cells

(21). To rule out these explanations, a kanamycin inhibition assay was performed on uninduced cells. The survival rate of uninduced *E. coli* cells (i.e. cells without osmotic stress induced capsule formation) exposed to kanamycin at different NaCl concentrations showed similar decrease in survival rate (Fig. 4). The turbidity reading results from this assay provided empirical support that the NaCl concentrations tested in this study did not have an inhibitory effect on the activity of kanamycin. Therefore, the increased survival observed at higher concentrations of NaCl was likely due to changes in the bacteria, possibly from protection provided by the induced capsule rather than a reduction in kanamycin activity. A caveat of using the turbidity assay to assess growth inhibition was that intact inhibited cells could still scatter light, even though they could no longer divide, and yielded relatively high turbidity readings at high kanamycin concentration (3.0 µg/ml). It was due to time constraints that plate counts could not be used to assess the effect of NaCl on kanamycin activity. In summary, the data suggested that the greater capsular polysaccharide production was induced by higher NaCl osmotic pressures and subsequently this capsule rendered better protection against kanamycin (Fig. 3).

A previous study by Drayson *et al*. showed that kanamycin and streptomycin resistance was independent of capsule size by comparing survival of wild type as well as mutant *E. coli* strains that overproduced and underproduced capsules (2). Their conclusion seems to contrast with what was observed in this study. However, this could be due to the fact that with osmotic pressure, polysaccharide composition of the cell capsules was altered without increasing the size of the capsule, although capsular size was not directly assessed in this study. On the other hand, another study done in 2008 by Lu *et al.* provided evidence for the effectiveness of stress exposure in *E. coli* leading to larger capsule size and resulting in the higher survival of the cells in a second stress condition, when compared to cells with no previous exposure (12). Additionally, the study by Liu *et al.* also supported the results of this study, showing that capsule sizes after stress exposure were significantly higher than those of normally incubated cells, and subsequent exposure to kanamycin increased survival rate (11).

The results of this study reveal that production of capsule-associated carbohydrates was induced at higher NaCl osmotic pressure as indicated by the increase in glucose and fucose production, and the cells were better able to survive with kanamycin exposure. The data addressed the experimental question that high concentrations of NaCl can induce capsule production in *E. coli* B23 cells and render increased protection against kanamycin.

FUTURE DIRECTIONS

To address conflicting results from previous studies (2, 10, 12), capsule could be induced by means that would not interfere with the mechanism of action and activity of antibiotics used. Using an uncharged antibiotic to test protective effects of ion-induced capsule may help resolve charge-related antibiotic interference issues. Furthermore, this study could not separate intracellular polysaccharides from extracellular polysaccharides, even though under high osmolarity conditions, trehalose and sucrose can both be used as compatible solutes to increase intracellular concentration to prevent cell death by plasmolysis and contribute to the observed increase in glucose as measured by the anthrone assay (10). Using absorbance readings of carbohydrate assays that detects the presence of capsular components, such as hexuronic acids (17), that are strictly found in the capsule as an indicator of capsule size will help to reduce uncertainties resulting from potential cross-contamination with intracellular components.

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