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Capsular Polysaccharide Production in *Escherichia coli* K-30 Strain E69 May Increase Following Pretreatment with Tetracycline but not Polymyxin B

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The group 1 capsule is an extracellular polysaccharide layer that encapsulates *Escherichia coli* K30 strain E69. Capsule synthesis and transport is dependent on the Wzy biosynthesis system, which is up-regulated when *E. coli* cells are exposed to sub-minimum inhibitory concentrations of various antibiotics. In this study, we examined the effects of pretreatment with different classes of antibiotics on capsular polysaccharide (CPS) production in *E. coli* strain E69. We hypothesized that membrane-disrupting antibiotics like polymyxin B would cause greater osmotic stress and hence a greater increase in CPS production relative to antibiotics like tetracycline that disrupt protein synthesis. Subinhibitory concentrations of tetracycline and polymyxin B were used to pretreat strain E69 and *E. coli* strain CWG655Δ[*wza-wzb-wzc*$_{K30}$], which lacks 3 genes belonging to the Wzy system. Following pretreatment, CPS was extracted and quantified using the phenol sulphuric acid colourimetric assay. We observed that tetracycline exposure led to increased CPS production in E69 while polymyxin B exposure did not. Our preliminary data suggest that CPS production does not increase following pretreatment with antibiotics that disrupt the outer membrane but increases following pretreatment with antibiotics that inhibit translation. This study adds to our knowledge of factors that may contribute to bacterial resistance to antibiotics.

The capsule is a layer that encapsulates various bacterial species and is composed of a type of extracellular polysaccharide (EPS) called the capsular polysaccharide (CPS) (1, 2). Bound to the outer membrane, capsule serves a variety of functions, including: immune evasion by mimicking host cell antigens, masking bacterial antigens, and as a defense mechanism against desiccation by absorbing large amounts of water from the surroundings (3).

Capsules are categorized into four groups based on the genes regulating expression of both CPS and the capsule biosynthesis proteins and also based on the system of CPS translocation to the extracellular environment (4). The characteristics of the group 1 capsule and its biosynthesis and translocation pathway have been extensively studied (4, 5, 6). Group 1 capsules are synthesized and transported to the cell surface via the Wzy-dependent biosynthesis system which is comprised of Wza, Wzb, and Wzc, as shown in Figure 1 (4, 6). Wza is a membrane channel that translocates CPS and EPS across the outer membrane in *E. coli* (4). Wzc is a tyrosine autokinase located in the cytoplasmic membrane that is thought to be necessary for polymerization of high molecular weight CPS (4). Wzb is a phosphatase that inhibits Wzc activation (4). Deletion of these Wzy system genes results in reduced capsule synthesis and CPS translocation to the extracellular environment (6).

Under optimal conditions, group 1 capsule genes are constitutively expressed (6). However, according to Sailer *et al.*, environmental stresses such as osmotic stress up-regulate CPS production in *E. coli* that acts as a physical barrier to limit excess water movement across the cell membrane (7, 8). Similar to this response caused by osmotic stress, exposure of *E. coli* to sub-minimum inhibitory concentrations (sub-MIC) of kanamycin, streptomycin, and cephaloridine induces 10% to 41% increase in capsule production, although the mechanism is not well understood.

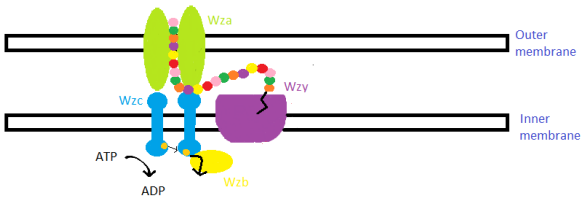

FIG 1. A modified model of the group 1 capsule Wzy-dependent biosynthesis and transport system including Wza, Wzb and Wzc. Capsular polysaccharide (CPS) repeats are synthesized in the cytosol and flipped across the inner membrane (6). Wzy is a polymerase that extends these repeats into high molecular weight CPS with help from Wzc, which is a tyrosine autokinase that is negatively regulated by Wzb phosphatase (6). These polysaccharides are then transported through the outer membrane channel, Wza, to the cell surface (6).

(9, 10). Ophir *et al.* showed a greater increase in expression of CPS producing genes in *E. coli* upon exposure to sub-MIC of antibiotics that directly compromise membrane integrity compared to other antibiotic classes (3). A potential explanation for this phenomenon is that the exposure to membrane altering antibiotics can lead to greater osmotic stress (7).

In this study we compared capsule production following exposure to antibiotics that target either the cell wall or translation. We hypothesized that exposure of *E. coli* K30 strain E69 (E69) to antibiotics that directly compromise membrane integrity would induce greater CPS production than antibiotics that inhibit translation since increased CPS production has been identified as a protective response to greater osmotic stress caused by membrane disrupting antibiotics (7). We analyzed *E. coli* strain E69 strain, a well-established model system used to study the group 1 capsule and its Wzy biosynthesis pathway (4). Contrary to our hypothesis, our data suggest that treatment with the

membrane targeting polymyxin B does not up-regulate capsule production but treatment with the aminoglycoside tetracycline does.

MATERIALS AND METHODS

Bacterial strains, media preparation, and E69 strain conditions *E. coli* K30 strain E69 (serotype O9a:K30:H12) and CWG655 (genotype wza22 min::*aadA* Δ(*wza-wzb-wzc*) K30::aphA3 Kmr Spr) were obtained from MICB 421 culture collection (Department of Microbiology & Immunology, University of British Columbia (UBC)). Overnight cultures of E69 strain and CWG655 mutant were prepared by inoculating 5 ml of Luria-Bertani (LB) medium (1% Tryptone, 0.5% yeast extract, 0.5% NaCl) in 16 x 125 mm test tubes with a loop-full of the respective strain from streaked plates. The inoculated cultures were grown overnight on a shaker at 100 rpm at 21^0C since Botros *et al*. found higher CPS production at this temperature (1).

Sample preparation and imaging of the E69 and CWG655 capsule using transmission electron microscopy Samples were prepared according to the protocol explained by Karlyshev *et al.* and the personnel at the UBC bio-imaging facility performed the steps of the assay (11). Overnight cultures of E69 strain and CWG655 mutant were standardized to OD_{600} of 2.0 and centrifuged for 5 minutes at 14,600 x g to obtain a cell pellet. After the supernatant was discarded, the cells were re-suspended in 3% glutaraldehyde prepared in 0.075 M sodium carbohydrate buffer (pH 7.4) with 0.1% ruthenium red (capsule specific stain) for 2.5 hours. The samples were then exposed to 1% osmium tetroxide for 1.5 hours to fix membrane lipids and subsequently washed with ultrapure MilliQ water. The samples were dehydrated through exposure to a graded series of ethanol from 30% to 100% with 10 minutes of incubation at each step. The samples were then transferred sequentially into 3:1, 1:1 and 1:3 ethanol-TAAB resin mixtures for 1 hour per step and were finally transferred into 100% TAAB resin overnight. Samples were then incubated in fresh TAAR resin for 3 hours and embedded in copper moulds. The blocks were removed from moulds and cut into 100 nm thick sections using a glass knife and Leica Ultracut R ultramicrotome. The sections were then placed into copper grids and were stained with 2% alcoholic uranyl acetate for 10 minutes in the dark. Then the samples were thoroughly washed in MilliQ, stained for another 10 minutes on a drop of lead citrate, washed and allowed to air dry before imaging using the Jeol 1200EX transmission electron microscope, XR51camera at 80.0 kV. Electronic images were obtained for E69 strain and CWG655 samples.

The minimum inhibitory concentration (MIC) assay The MIC assay protocol was adopted from Dhanoa *et al* (9). Overnight cultures of E69 strain and CWG655 mutant were grown at 21°C on a shaker at 100 rpm and were standardized to 0.5 OD_{600} post incubation to obtain a concentration of cells in the range of 10^6 cell/mL. Two-fold serial dilutions were made in a Falcon 353072 96-well plate to obtain wells containing 8 µg/ml, 4 µg/ml, 2 µg/ml, 1 µg/ml, 0.5 µg/ml, 0.25 µg/ml, and 0 µg/ml (control) of polymyxin B (polymyxin B sulphate salt) or tetracycline (tetracycline hydrochloride salt). Each well was inoculated with 10 µl of 1/40 dilutions of E69 strain or CWG655 mutant. Sterile LB media wells were used as negative control, while the positive controls comprised of E69 and CWG655 strains without antibiotic treatments and were used to compare turbidity between wells. The well with the lowest concentration of antibiotic that did not show growth upon visual observation indicated the MIC.

Sub-minimum inhibitory antibiotic pre-treatment. Sub-MIC was calculated as half the concentration of the previously determined MIC values. 60 µl of diluted overnight culture (0.5 OD_{600}) of E69 strain and CWG655 strains was added into tubes with LB to a total volume of 5 ml containing sub-MIC of polymyxin B and tetracycline. All 4 cultures were incubated for 24-48 hours at 21°C with a shaker setting at 100 rpm until turbidity was observed.

Surface polysaccharide extraction assay. Surface polysaccharides were extracted using a modified method adapted from Brimacombe and Beatty (12). Overnight untreated samples, as well as sub-MIC antibiotic pretreated samples of E69 strain and CWG655 mutant were standardized to 2.0 OD_{600}. 1ml of each sample was centrifuged at 14,600 x g for 5 minutes using an Eppendorf 5415D Microcentrifuge. The same centrifugation conditions were used for all subsequent centrifugation steps. After the supernatant was discarded, the pellet was re-suspended in 1 ml of 50 mM EDTA and incubated at 37°C for 1.5 hours while on a 150 rpm shaking rack. This step dissociates LPS and releases the outer membrane from cells. The samples were then centrifuged and the supernatant containing the capsular polysaccharides (CPS) was extracted for the quantification assay.

Surface polysaccharide quantification assay. Surface polysaccharides obtained using the extraction assay were quantified using a phenol-sulphuric acid assay adopted from Dhanoa *et al*., which was modified in accordance with Masuko *et al* (9, 13). Carbohydrate standards were prepared by diluting 1mg/ml of sterile stock carbohydrate solution (1:1 solution of sucrose and fructose dissolved in distilled water with a final carbohydrate concentration of 1 mg/ml) into 1 ml aliquots with final concentrations of 0, 10, 20, 30, 40, and 50 µg/ml. 800 µl of each carbohydrate sample, and 50 mM EDTA (negative control) was transferred into a clean glass test tube. 4 ml of 95% H_2SO_4 was added to each sample, immediately followed by addition of 800 µl of 5% phenol (prepared ~ 6 minutes before use). The samples were then gently vortexed and incubated in stationary water baths at temperatures of 90°C for 5 minutes followed by another 5 minutes at 21°C to allow for colour development. Colour intensity for each sample was immediately measured following the 10-minute incubation. The 0 µg/ml was used as a blank when taking measurements at 490 nm using the Spectronic 20^+ spectrophotometer. Absorbance values of extracted samples were then normalized to the carbohydrate standards to calculate the CPS concentration.

RESULTS

Transmission electron microscopy shows greater capsule production in E69 strain compared to CWG655 mutant. In order to visualize capsule we compared strain E69 and the isogenic capsule deficient strain CWG655(Δ*wza-wzb-wzc)* using transmission electron microscopy (TEM). Strain E69 and CWG655 were grown in LB liquid media at 21°C on a 100 rpm shaker for 24 hours and were then processed for TEM imaging. During fixation, the cells were stained with ruthenium red dye, which specifically stains capsule (11). Several 100 nm thick cross sections of stained E69 and CWG655 cells were imaged at magnifications ranging from 70,000x to 100,000x (Fig. S1).

Figure 2A shows a representative cross section of strain E69. Thread-like structures protruding from the outer membrane are visible which we interpret to be extracellular capsule. Figure 2A also shows extracellular capsule, which appears to be distributed as tufts of varying thickness around the cell surface.

Figure 2B shows a representative cross section of the capsule transport protein deficient mutant strain CWG655 (Δ*wza-wzb-wzc*). CWG655 has been shown to produce low

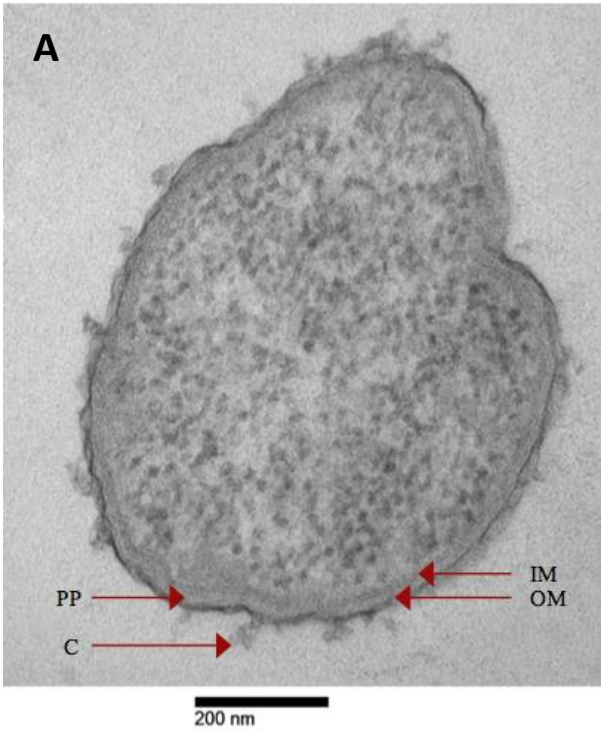

FIG 2. TEM images of E69 strain (A) and CWG655 mutant (B) stained with ruthenium red dye. IM refers to inner membrane, OM refers to outer membrane, PP refers to periplasm, and C refers to ~20 nm (scale-bar approximation) long thread-like structures interpreted as the capsule. 100 nm thick cross sections of cells were imaged at 100,000x original magnification at 80.0 kV.

levels of capsule (6). Figure 2B shows minimal thread-like structures protruding from the outer membrane compared to strain E69 (Figure 2A) Taken together these data suggest that strain E69 produces more capsule than strain CWG655, which is consistent with reported phenotype for this strain (6).

Biochemical analysis shows higher carbohydrate production in strain E69 compared to strain CWG655. In order to quantitatively measure the extracellular carbohydrate on strain E69 and strain CWG655, we extracted surface associated carbohydrates and measured its concentration using the phenol sulphuric acid assay (12). In this assay, sulphuric acid degrades the extracted polysaccharides into monosaccharides and phenol reacts with these monosaccharides to produce a yellow colour (9). This yellow colour has an absorbance maximum at 490 nm (A_{490}) and the intensity of colour produced is directly proportional to the concentration of carbohydrates in the sample (9). A standard curve was prepared using known concentrations of carbohydrate, (Supplementary data Fig. S2). CPS samples extracted from strain E69 and strain CWG655 were quantified using the phenol sulphuric acid assay and the standard curve was used to convert A_{490} values into corresponding carbohydrate concentrations. All measurements were normalized against optical density values to correct for differences in cell concentration.

Strain E69 had 25.2 µg/ml/OD_{600}, Strain CWG655 had 20.1 µg/ml/OD_{600}. Strain E69 strain had 1.3 fold (25.4%) higher concentration of carbohydrates (Fig. 3). This result is consistent with the results from TEM. The results of the phenol sulphuric acid assay suggests that strain E69 produces more extracellular carbohydrates compared to strain CWG655. We had now established a biochemical assay to measure extracellular carbohydrate production.

Minimum inhibitory concentration of polymyxin and tetracycline in strains E69 and CW655. Our goal in this study was to test the effect of antibiotic treatment on capsule production in strains E69 and CW655. In order to do this, we needed to pretreat strain E69 and CWG655 with sub-MICs of each antibiotic. In order to determine the sub-MIC of E69 strain and CWG655 mutant for polymyxin B and tetracycline, we conducted an MIC assay. Cells were incubated with different concentrations of antibiotics to determine the minimum concentration of antibiotic that inhibits cell growth. This antibiotic concentration represents the MIC. Strains E69 and CWG655 that were incubated in the absence of antibiotic were used as a positive control for growth. Sterile LB media served as a negative control.

MIC of polymyxin B was identical for strain E69 and strain CWG655 at 0.50 µg/ml The sub-MIC of strain E69 and strain CWG655 for tetracycline was 0.25µg/ml and 1.0 µg/ml, respectively. The sub-MIC for each treatment was set at half the MIC and is shown in table 1 (9). The results indicate that strain E69 and CWG655 mutant are equally sensitive to the effects of polymyxin B while strain E69 is more sensitive to tetracycline than CWG655.

Production of EPS in strain E69 increases following pretreatment with tetracycline but decreases following pretreatment with polymyxin B. In order to determine the

Table 1: Subminimum inhibitory concentrations of polymyxin B and tetracycline for treatment of *E. coli* strains E69 and CWG655 mutant

Strain name	Sub-MIC for polymyxin B (µg/ml)	Sub-MIC for tetracycline (µg/ml)
E 69 strain	0.25	0.12
CWG655 mutant	0.25	0.50

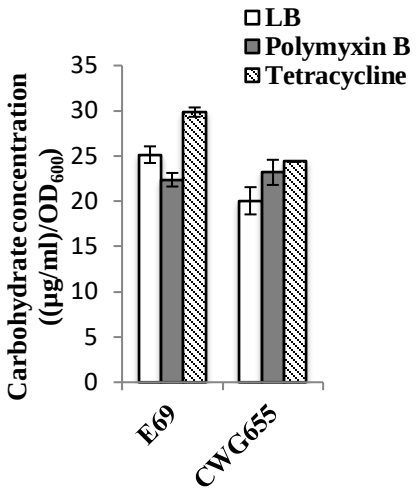

FIG 3. Effect of sub-MIC treatment with polymyxin B and tetracycline on extracellular carbohydrate production in *E. coli* E69 strain and CWG655 mutant. The strains were grown in 21^{0}C on 100 rpm shaker for 24-48 hours with or without antibiotic pretreatment until turbidity was observed. Extracellular carbohydrates were extracted for each sample and quantified using the phenol sulphuric acid assay. Absorbance values were then normalized using the standard curve and OD_{600} readings. The error bars represent standard deviation calculated over 3 technical replicates.

effect of exposure to different classes of antibiotics on extracellular carbohydrate production in strain E69, we exposed cells to sub-MIC levels of polymyxin B or tetracycline. We then quantified the amount of carbohydrate produced for each treatment using the phenol sulphuric acid assay. Polymyxin B was chosen since it directly alters membrane integrity, which may impact the osmotic stress response. Tetracycline was chosen since it inhibits translation and does not directly impact membrane integrity (14, 15). Untreated E69 and CWG655 samples were used as controls to establish a baseline value for each strain in terms of carbohydrate production. Since the CWG655 mutant produces low amounts of extracellular carbohydrate it was used as a negative control (9).

Exposure of strain CWG655 to sub-MIC levels of polymyxin B and tetracycline led to a 1.2 fold (18.7%) increase in extracellular carbohydrate production compared to the untreated CWG655 control (Fig 3). Exposure of strain E69 to sub-MIC levels of polymyxin B led to a 1.1 fold (12.3%) decrease in concentration of carbohydrates produced by strain E69 compared to the untreated E69 control. Exposure of strain E69 to sub-MIC levels of tetracycline showed a 1.2 fold (15.7%) increase in concentration of carbohydrate production when compared to the untreated strain E69 control (Fig. 3).

Exposure of strain E69 to sub-MIC levels of tetracycline increases extracellular carbohydrate production while exposure to sub-MIC levels of polymyxin B does not. Taken together, these preliminary data suggest that pretreatment of strain E69 with sub-lethal levels of tetracycline leads to a modest increase in capsule production while, pretreatment with polymyxin B leads to a slight decrease in capsule production.

DISCUSSION

Capsule is an extracellular layer of polysaccharides that provides protection from various environmental stresses such as exposure to antibiotics (3, 16). We studied changes in extracellular carbohydrate production post treatment with different classes of antibiotics on *E. coli* K30 strain E69. We first used TEM to qualitatively observe capsule production by CWG655 and E69. We then used a phenol sulphuric acid assay to quantitatively measure extracellular carbohydrate concentration. This assay was then used to analyze the effects of different classes of antibiotics on capsule production.

TEM images of multiple sections showed capsule in the form of thread-like structures protruding from the outer membrane (Fig. 2, Fig. S1). E69 and CWG655 cells were prepared using the same protocol, but only E69 cells were observed to have these protruding structures (Fig. 2). This observation suggests that the structures seen in E69 TEM images were not an artifact of the TEM processing and represented cellular capsule. We observed that more capsule was produced by E69 compared to strain CWG655 (Fig. 2A, 2B). These results were expected since the CWG655 mutant lacks *wza*, *wzb* and *wzc* genes, which are important for CPS transport to the cell surface (6). Even though Whitfield observed a similar trend i.e., a higher CPS production in E69 compared to CWG655 mutant, however, they observed a thicker capsule surrounding E69 cells in comparison to our observations (4). This difference could be explained by the fact that Whitfield used a ferritin-staining procedure, while we used ruthenium red dye to stain capsule (4, Fig 2A). The positive charge of ferritin stabilizes the negatively charged capsule, which prevents its loss during cell processing (6). Ruthenium red provides no such stabilization, which may explain the decrease in amount of capsule seen in our TEM images of strain E69 cells (11, Fig. 2).

Consistent with our TEM results, the phenol sulphuric acid assay also showed that E69 cells produced more extracellular polysaccharide compared to strain CWG655 (Fig. 3). Using this assay, Parmar *et al.* observed a greater extracellular polysaccharide production in the *E. coli* strain K12 (wildtype) compared to the Δ*wza* mutant (18). However, they observed a 10-fold difference in EPS production between wildtype and

mutant strains, while our results showed only a 1.3 fold difference (18) (Fig. 3). The use of different strains of *E. coli* cells may explain this difference; since Parmar *et al.* used strain K12 that produces group 2 capsule, whereas we used strain E69 that produces group 1 capsule (4). One of the components of group 2 capsules is colanic acid, which is produced in large amounts, but is lacking in group 1 capsule (4). This over production of colanic acid in K12 may explain the large fold difference found by Parmar *et al*. since the sulphuric acid assay might be more sensitive to colanic acid when compared to other polysaccharides (4, 18).

Extracellular carbohydrate production was then studied after pre-treatment with different classes of antibiotics on E69 and CWG655. Polymyxin B belongs to a class of antibiotics that disrupt membrane integrity (22). Prior studies suggest that pretreatment with polymyxin B induces osmotic stress and increases CPS production (2, 7, 20). However, we observed a decrease in extracellular carbohydrate production in E69 cells upon exposure to polymyxin B (7, 22) (Fig. 3). This appears to contradict the notion that the E69 cells increase capsule production as a protective response to reduce osmotic stress (2, 7, 20). The effect of growth phase on capsule production in *E. coli* might provide an explanation for this inconsistency (21). Polymyxin B pre-treated E69 cells grew to an observable turbidity 3 days after inoculation, whereas the untreated cells reached this state within 12 hours. This extended period of growth may have led the pre-treated E69 cells to reach stationary phase where carbohydrate production has been shown to be subdued (21).

In contrast, polymyxin B pre-treated CWG655 displayed greater CPS than its E69 counterpart (Fig. 3). Polymyxin B treated CWG655 grew one day sooner than polymyxin B treated E69. As a result, the polymyxin B treated mutant's extracellular carbohydrates may have been extracted in exponential growth phase, which is the optimal stage for capsule expression (21). This would explain why the polymyxin B treated E69 showed lower extracellular carbohydrate levels than polymyxin B treated CWG655 (Fig. 3). Figure 3 also shows error bars representing standard deviation over 3 technical replicates for all treatment groups. The error bars for CWG655 samples were twice as large as those for E69 samples (Fig. 3). This higher variability in CWG655 results might suggest that the fold difference seen between polymyxin B treated CWG655 and strain E69 is an overestimation of the actual fold difference between the two.

Tetracycline belongs to a class of antibiotics that inhibits protein synthesis by blocking interaction of the aminoacyl-tRNA with the 30S ribosomal unit (15). As indicated in Figure 3, concentration of extracellular polysaccharides increased for both strains after pre-treatment with tetracycline. The action of tetracycline may result in scarcity of structural proteins in the outer membrane, rendering it weak (7). Weak outer membrane leaves these cells prone to osmotic stress causing increased CPS production in both E69 and CWG655 (2). Similar effects were observed in other *E. coli* strains when exposed to kanamycin and streptomycin, both of which inhibit protein synthesis (2, 19). Thus, the increase in capsule observed in Figure 3 in E69 and CWG655 strains may serve as a protective mechanism against osmotic stress induced by tetracycline by absorbing large amounts of water. In hypertonic conditions, cells are prone to desiccation and the capsule can help reabsorb water to prevent this from happening (3). In hypotonic conditions, cells are at risk of lysis from rapid inflow of water; this can also be prevented by the action of capsule where CPS absorbs the extraneous water (3).

In conclusion, results from both TEM images and carbohydrate quantification assay suggest greater CPS production in strain E69 versus CWG655 mutant. We also found that pre-treatment with tetracycline led to an increase in CPS production. However, pre-treatment with polymyxin B did not lead to an increase in CPS production. Our data suggest that exposure to translation-inhibiting antibiotics such as tetracycline may increase CPS production.

FUTURE DIRECTIONS

Strain E69 and CWG655 mutant were exposed to two antibiotics belonging to different classes to determine their effect on CPS production. It is difficult to make predictions about the behavior of entire antibiotic classes based on the use of one representative from each class. In order to be able to compare the effects of entire antibiotic classes, we suggest exploring the effects of other antibiotics belonging to the classes used in this study. This project could also be approached using TEM where cells are exposed to sub-MIC of various antibiotics and imaged to evaluate differences in CPS production.

Further studies could investigate the effect of increased CPS production on antibiotic resistance following sub-MIC pretreatment. To this end, MIC assay would be used to determine the susceptibility of strain E69 to various antibiotics. Strain E69 could be treated with sub-MIC of antibiotics, followed by redetermination of the MIC. These results, when compared to the MICs for untreated samples, would indicate changes in the functional resistance to these antibiotics. Increasing levels of MIC would signify increased resistance, which can be correlated with the role of capsule in providing resistance when compared to the results for a capsule deficient control strain.

Strain E69 produces group 1 capsule and does not involve production of colanic acid. However, capsule groups 2, 3, and 4 produce large quantities of colanic acid, which explains the higher CPS production seen in other strains of *E. coli* when compared to strain E69. A future direction would include comparing CPS produced by *E. coli* wild type strains and those that have knockouts in the gene

responsible for production of colanic acid. This comparison would elucidate the contribution made by colanic acid to concentration of CPS found in these strains.

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