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The Role of *wza* in Extracellular Capsular Polysaccharide Levels During Exposure to Sublethal Doses of Streptomycin

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It has been previously shown that treatment of *Escherichia coli* with streptomycin triggers an increase in capsular polysaccharide (CPS) production. In this study, we investigated whether an increased export of CPS to the cell surface and therefore a thicker CPS is correlated with streptomycin resistance. This was done by using a strain lacking *wza* which codes for a protein involved in the transport of CPS polymers to the cell surface. The results of the anthrone assay revealed that extracellular CPS levels following stimulation with sublethal concentrations of streptomycin was lower for the Δ*wza* strain compared to the wild-type (WT) strain indicating that exposure to the antibiotic does indeed induce CPS production and export and also confirmed the role of *wza* in CPS transport. Furthermore, it was seen that WT treated cells were more resistant to the lethal antibiotic concentrations indicating a role for the thicker CPS in antibiotic resistance. However, the Δ*wza* strain did not display a lower level of resistance to antibiotics.

Most Gram-negative bacteria, the best characterized being *Escherichia coli,* constitutively produce extracellular polysaccharide which is either secreted as a viscous fluid product or forms the bacterial capsule on the cell surface (9). The bacterial capsule is composed primarily of K antigens and O antigens, which resemble lipopolysaccharides (9). Each class of antigens has many different serotypes due to differential sugar composition and linkage specificities (8). K antigens are divided into two groups, group I and group II, based on their serological properties (8). The hydrophilic nature of the bacterial capsule facilitates its many functions, including protecting the bacterium from desiccation in addition to phagocytosis by host immune cells, thus providing a physical barrier against bacteriophage infection, promoting surface adhesion and subsequent biofilm formation (8). Most relevant to this current study is the fact that the polysaccharide capsule of Gram-negative bacteria confers protection, through several putative mechanisms, from the action of certain antibiotics (8). One mechanism is based on the principle that the length of time required for the diffusion of the antibiotic is proportional to the thickness of the extracellular capsule. In other words, thicker polysaccharide capsules effectively retard the diffusion of the antibiotic across the capsule and subsequently into the cell. The alternate mode of action is based on electrostatics. The electrostatic attraction between the negatively-charged capsule and positively-charged antibiotics, namely aminoglycosides like streptomycin, effectively prevent the diffusion intracellularly of the respective antibiotic (8).

A study by Ganal *et al*. demonstrated that subjecting *E. coli* B23 to sublethal doses of either kanamycin or streptomycin resulted in subsequent increased production of CPS (5). The results obtained by Lu *et al*. revealed the correlation between increased CPS production and antibiotic resistance in *E. coli* B23 cells (5). Furthermore, Himaras *et al*. investigated the physiological role of *cpsG,* which encodes the protein CpsG involved in colanic acid synthesis, and noted that exposure to sublethal doses of aminoglycosides still resulted in upregulated CPS levels (4) even though colanic acid is one of the exopolysaccharide constituents of the capsule (3, 4).

In this study, the role of *wza*, an alternative gene implicated in CPS translocation, was investigated, since it had been demonstrated by Dong *et al*. that the knockout of *wza* precludes capsule formation (2). Its respective protein product, Wza, is involved in the export of group I polysaccharides in *E. coli* (2).

We examined whether the *wza* translocon was implicated in increased CPS production following exposure to streptomycin and the subsequent conferral of increased antibiotic resistance capabilities. This hypothesis was tested through the comparison of the turbidity readings at λ = 460nm at varying concentrations of streptomycin in addition to the quantification of CPS production between the *wza* mutant and WT strains.

MATERIALS AND METHODS

Bacterial strains. The *Escherichia coli* strains used in this experiment were provided by the Coli Genetic Stock Center at Yale

University. The strains used were JW2047-1 (genotype: *F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), LAM-, Δwza-760::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514*) and BW25113 (genotype: *F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), LAM-, rph-1, Δ(rhaD-rhaB)568, hsdR514*). In this article, JW2047-1 will be referred to as Δ*wza* strain and BW25113 as the wild-type (WT) strain. JW2047-1 and BW25113 are genetically identical except for the absence of *wza* gene and the presence of a kanamycin resistance gene in JW2047-1. A colony from a freshly streaked JW2047-1 plate was then selected and plated on a streptomycin containing LB plate (50 µg/ml, LB containing 1.08% Agar) and incubated overnight at 37 ºC to check for any cross-reactivity due to the kanamycin resistance gene.

Growth of *E. coli* JW2047-1 and *E. coli* BW25113. Overnight cultures was prepared by inoculating 100 ml of LB medium (1% Tryptone, 0.5% Yeast Extract, 0.5% NaCl, 0.4% Glucose) with a loopful of the respective strains. The inoculated cultures were placed on the shaker at 200 rpm in the 37ºC incubator overnight. 15 ml of each overnight culture were then used to inoculate two flasks with 175 ml of LB media. The newly inoculated cultures were then grown in a shaking water bath at 200 rpm for 1 hour at 37ºC.

Sublethal streptomycin treatment. Streptomycin sulfate (IGN-#100556) was dissolved in sterile distilled water to a final concentration of 20 mg/ml for use as a stock solution and was stored at -20ºC. Streptomycin stock solution (20 mg/ml) was added to one flask of 190 ml WT inoculated culture (15 ml inoculum + 175 ml LB medium) and one flask of 190 ml Δ*wza* inoculated culture to create a 10 µg/ml pretreatment condition. Equivalent LB medium was added to one flask of 190 ml WT inoculated culture and one flask of 190 ml Δ*wza* inoculated culture to serve as control conditions. All four cultures were then incubated in a shaking water bath at 200 rpm at 37ºC for an additional hour to induce increased capsular polysaccharide production. Subsequently, turbidity was measured using Spectronic 20+ spectrophotometer set at wavelength 460 nm, using LB medium as blank.

Streptomycin antibiotic resistance test. To 16 x 125 mm glass test tubes, sufficient volumes of streptomycin stock solution (5 mg/ml) were added to create final streptomycin concentrations of 20, 25, 30, 40, 50, 75, 100, 125, 150, and 175 µg/ml for 5 ml total volume. Four sets of these antibiotic resistance tubes were prepared to test the streptomycin pretreated WT, control WT, streptomycin pretreated Δ*wza*, and control Δ*wza*. LB medium was added to bring the final volume to 3.8 ml. 1.2 ml of each of the four respective bacterial cultures from the 190 ml cultures were added to corresponding test tubes. Four controls were then set-up accordingly: streptomycin pretreated WT, control WT, streptomycin pretreated Δ*wza*, and control Δ*wza* in tubes with no streptomycin. Again, this was prepared in similar fashion to the above, 1.2 ml of culture in 3.8 ml of LB medium. The controls provided an approximate baseline value for each cultures growth in non-antibiotic tubes and also allowed for comparison between each. Samples were then incubated overnight at 37°C. Turbidity of the samples was read using the Spectronic 20+ spectrophotometer with wavelength set at 460 nm, using LB medium as blank.

Capsule isolation. 120 ml of each culture (streptomycin pretreated WT, control WT, streptomycin pretreated Δ*wza*, and control Δ*wza*) was centrifuged using a Sorvall RC-5B Refrigerated Superspeed Centrifuge using the SLA 1500 Super-Lite rotor 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 4.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4) to ¼ of the original volume. PBS resuspended pellets were blended in an Osterizer 8300 Series Waring blender for 5 min at the frappe setting. Exopolymers were then precipitated by adding two volumes of ice-cold acetone. The resulting suspension was centrifuged using a Sorvall RC-5B Refrigerated Superspeed Centrifuge using the SLA 1500 Super-Lite rotor 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 placed in Spectra/Por® molecularporus membrane dialysis tubes with a molecular weight cut-off of 6,000-8000 kDa. These samples were dialyzed against 1.8 L of distilled water at room temperature for 24 hours. The dialyzed exopolymers were dried overnight using a lyophilizer . The dried crude exopolymers were dissolved in 20 ml of sterile 10 mM $MgCl_2$. Deoxyribonuclease I (DNase I) (Sigma #2326670) and ribonuclease A (RNase A) (Sigma R-4875) 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 200 rpm 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 Sorvall RC-5B Refrigerated Superspeed Centrifuge using the SLA 1500 Super-Lite rotor 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 Sorvall RC-5B Refrigerated Superspeed Centrifuge using the SLA 1500 Super-Lite rotor 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 200 mg of anthrone (MC&B-#AX1655) in 5 ml of 100% ethanol. The solution was then made up to 100 ml using concentrated (93%) sulfuric acid (JT Baker) and chilled until used. Duplicate of half millilitre samples of each purified capsule sample were placed into glass test tubes and chilled on ice for 2 to 3 min. 2.5 ml of anthrone reagent was added to the samples in the chilled test tubes, and carefully vortexed. The tubes were then transferred to a boiling (100°C) stationary water bath and capped with glass marbles. Tubes were incubated for exactly 10 min and then transferred back to ice to cool and stop the reaction. Once cooled, 3 ml volumes of samples were read at λ = 625 nm using a Spectronic20D spectrophotometer. Glucose equivalent standards ranging from 0 µg/ml to 100 µg/ml were prepared using α-D(+) glucose (Sigma #G-5000) diluted with dH_2O. Standards were treated the same way as test samples, and a standard curve was generated.

RESULTS

Antibiotic resistance test. The general trends demonstrated that the streptomycin-treated WT had higher turbidity readings at λ = 460 nm than their control (Fig. 1). This indicates that the streptomycin-treatment was sufficient to induce CPS production and confer antibiotic resistance. Another trend observed was that *wza* control was higher than *wza* strep, except at concentrations 75 µg/ml and 175 µg/ml. Since the *wza* strain is lacking the transport system for CPS export, this result reflects the role of the extracellular CPS in conferring antibiotic resistance. Surprisingly, *wza* LB was higher than WT control. Furthermore, as the antibiotic concentration is increased there is a general trend of decreasing turbidity values for all the samples (Fig. 1). This is expected because the antibiotic concentrations are increasing in lethality and it is possible that the CPS capsule is no longer sufficient in providing resistance and therefore more cells are killed. Data for streptomycin concentrations below 20 µg/ml did not produce significant differences in turbidity readings (results not shown). Samples were done in singles due to time constraints and therefore no

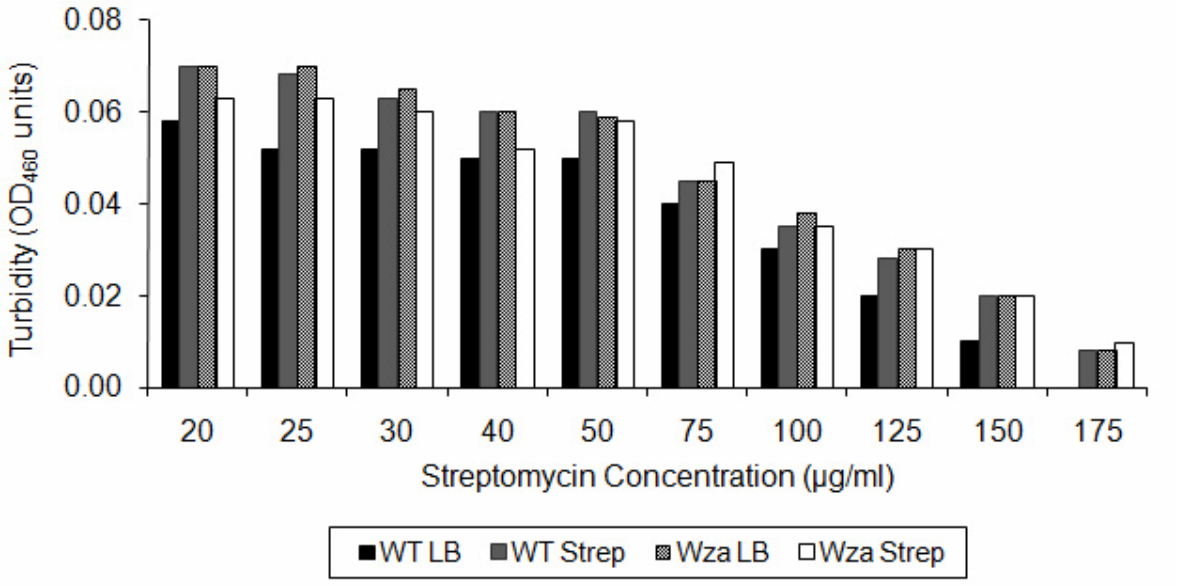

FIG. 1 Effect of streptomycin treatment between Δ*wza* and WT *E. coli* strains as represented by a streptomycin resistance test of concentrations ranging from 20 µg/ml to 175 µg/ml. The cells were treated in a condition of 10 µg/ml streptomycin and incubated at 37ºC for 1 hour (WT Strep, wza Strep). Controls were left untreated (WT LB, wza LB). In the streptomycin resistance tubes, the cells were grown in LB medium with varying concentrations of streptomycin. Incubation was done at 37ºC for 24 hours with shaking.

statistical analysis was performed.

Anthrone carbohydrate assay. The anthrone assay was done to measure the levels of extracellular polysaccharides which would be used to demonstrate the correlation between streptomycin resistance and increase CPS levels. The total glucose concentration was observed to be higher for the WT strains than the Δ*wza* strains (Fig. 2). This is expected because the levels of extracellular CPS are expected to be lower for the Δ*wza* strain as they lack the CPS export system. The results also indicate that antibiotic treatment does increase CPS levels. WT control produced CPS levels that were 1.25 times higher than the *wza* control, whereas WT strep showed CPS levels which were 1.6 times higher than *wza* strep. Samples done in singles due to time constraints.

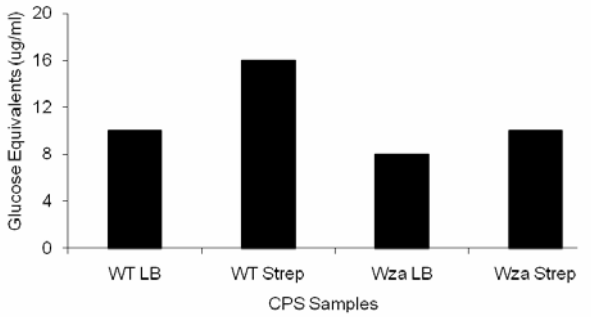

FIG. 2. Comparison of the effect of streptomycin treatment on the total capsular polysaccharide between Δ*wza* and WT *E. coli* strains. The treatments were carried out at a streptomycin concentration of 10 µg/ml (chosen based on the previous studies and to ensure consistency) and incubated at 37ºC for 1 hour. Controls were left untreated.

DISCUSSION

Many types of bacteria produce an extracellular layer of polysaccharides known as the capsule which has an important role in the interaction of bacteria with their extracellular environment (1). Importantly, the capsule of pathogenic microbes allows them to evade certain components of the host's immune defence and is therefore rendered a virulence factor (1). *E. coli* produces more than 70 capsules w\ith unique polysaccharides , which are classified into group I or group II polysaccharides. Group I capsular polysaccharides of *E. coli* are assembled by a Wzy-dependant polymerization system in which Wza is required for the surface expression of CPS as multimeric complexes of Wza which are believed to provide a channel for polymer export (7).

The total capsular polysaccharide assay (Fig. 2) showed that WT and Δ*wza* strains of *E. coli* treated with streptomycin produced notably higher levels of CPS compared to their respective controls without treatment, consistent with the results obtained by Lu *et al*. and Ganal *et al* wherein they found that treatment with either kanamycin or streptomycin increased CPS production (3, 5). They also found a correlation

between an increase in CPS production and increased antibiotic resistance capabilities (5). Treatment at a sublethal concentration of streptomycin (10 μg/ml,) was expected to stimulate increased CPS production and subsequent transport to the cell surface (5). Furthermore, it has been previously hypothesized that the resistance to aminoglycoside antibiotics is due to the induction of CPS production and that the resulting thicker CPS capsule would impede the entrance of the antibiotics into the cell (3, 5). Furthermore, the WT treated strain produced a higher amount of CPS compared to the Δ*wza* treated strain (Fig. 2). This was consistent with expectations since the Δ*wza* strain lacks one of its transport systems for exopolysaccharides. This was, however, not expected to fully knockout production of CPS since there are other transport systems available, one of which is the ABC-2 transporter involved in the transport of group II K antigen transport to the cell surface (9).

For the antibiotic resistance tests, it was noted that streptomycin concentrations between 0.3 μg/ml to 10 μg/ml did not produce notable differences in the turbidity readings between the control and streptomycin treated WT and Δ*wza* samples. This was in accordance with our expectations as these concentrations are sublethal and thus would not significantly affect the cells. According to Fig 1 the lethal antibiotic concentration is 20 μg/ml because up until this point, no significant difference in the turbidity values are seen.

It was noted that for the WT strain, the treated sample produced higher turbidity readings in comparison to its control (Fig. 1). This was anticipated because, as previous studies have shown, treatment induces increased CPS production which provides the cells with a thicker capsule, which in turn impedes the antibiotic from entering the cell (5). In addition, since a capsule is predominantly anionic and aminoglycosides are cationic, there is an electrostatic attraction resulting in the antibiotic being trapped in the capsule (8).

With the Δ*wza* strain, we expected that treatment would not produce as high of an increase in extracellular CPS production compared to the WT strep because of the lack of the Wza transport system. As mentioned, some CPS was still expected to be produced as the ABC-2 transporter was still functional, but not as much as WT strep (9). Therefore, it was expected that the turbidity readings for the Δwza samples would be lower than those for the WT samples. This general trend can be seen when comparing the turbidity values for the WT treated samples to the Δ*wza* treated samples (Fig. 1). However, paradoxically, the Δ*wza* control samples produced equivalent or higher turbidity values than both the control and treated WT samples (Fig. 1). This could be due to the fact that the WT sample turbidity readings were taken first, and the Δ*wza* samples were left on the bench top and had more time to grow. It took approximately 1 hour time to record the WT turbidity readings meaning the Δ*wza* samples had an additional ~1 hour to grow.

It can be observed that Δ*wza* strep had lower turbidity readings than the control Δ*wza*, except at concentrations of 75 μg/ml and 175 μg/ml (Fig. 1). This was noted, and could have been due to experimental errors, but the general trend still shows that Δ*wza* strep had lower turbidity readings than the control. This was anticipated, as the Δ*wza* strain was missing one of the essential components implicated in exopolysaccharide transport and therefore would not have as thick of a capsule as its WT counterpart (9). Therefore, sublethal treatment of Δ*wza* might have caused an inhibition of growth as it has been shown that at low concentrations, streptomycin causes growth inhibition rather than bactericidal effects (6). If this is the case, then the treatment would have caused a reduction of growth of the cells, and subsequently lower turbidity readings in the antibiotic resistance tests (Fig. 1).

In conclusion, it was noted that treatment with sublethal concentrations of streptomycin resulted in greater extracellular CPS content. Furthermore, the CPS levels were higher in the WT compared to the Δ*wza*, which was expected as the Δ*wza* is deficient in a transport system. As per our hypothesis, it was anticipated that the lower levels of CPS in the Δ*wza* strain would correlate with a decreased streptomycin-resistant phenotype and thus lower survival rates. However, our data did not conclusively support this premise, therefore we cannot conclude that increased extracellular CPS production correlates with aminoglycoside antibiotic resistance.

FUTURE EXPERIMENTS

It would be important to mutate alternate pathways implicated in CPS production and exopolysaccharide transport pathways to assess the explanation of the observation that Δ*wza* strain still produced extra polysaccharide after treatment with streptomycin.

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REFERENCES

1. **Boulnois, G. J. and K. Jann.** 1989. Bacterial polysaccharide capsule synthesis, export and evolution of structural diversity. Mol. Microbiol. **3:**1819-1823.
2. **Dong, C., K. Beis, J. Nesper, A. L. Brunkan-Lamontagne, B. R. Clarke, C. Whitfield, and J. H. Naismith.** 2006. Wza the translocon for *E. coli* capsular polysaccharides defines a new class of membrane protein. Nature. **444:**226-229.
3. **Ganal, S., C. Gaudin, K. Roensch, and M. Tran.** 2007. Effects of streptomycin and kanamycin on the production of capsular polysaccharides in *Escherichia coli* B23 cells. J. Exp. Microbiol. Immunol. **11:**54-59.
4. **Himaras, Y., F. Lin, S. Kim, and P. Cao.** 2004. The Role of *cps*G in Capsular Carbohydrate Accumulation in Response to Aminoglycoside Stimulation. J. Exp. Microbiol. Immunol. **12:**45-49.
5. **Lu, E., T. Trinh, T. Tsang, and J. Yeung.** 2008. Effect of growth in sublethal levels of kanamycin and streptomycin on capsular polysaccharide production and antibiotic resistance in *Escherichia coli* B23. J. Exp. Microbiol. Immunol. **12:**21-26.
6. **Luzzatto, L., D. Apirion, and D. Schlessinger.** 1968. Mechanism of action of streptomycin in *E. coli*: interruption of the ribosome cycle at the initiation of protein synthesis. P. Natl. Acad. Sci. USA. **60:**873-880.
7. **Reid, A. N. and C. Whitfield.** 2005. Functional analysis of conserved gene products involved in assembly of *Escherichia coli* capsules and exopolysaccharides: evidence for molecular recognition between Wza and Wzc for colanic acid biosynthesis. J. Bacteriol. **187:**5470-5481.
8. **Slack, M. P. E. and W. W. Nichols.** 1982. Antibiotic penetration through bacterial capsules and exopolysaccharides. J. Antimicrob. Chemother. **10:**368-372.
9. **Whitfield, C. and I. S. Roberts.** 1999. Structure, assembly and regulation of expression of capsules in *Escherichia coli*. Mol. Microbiol. **31:**1307-1319.