The *Escherichia coli* K12 Capsule does not Confer Resistance to Either Tetracycline or Streptomycin

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Bacterial capsules can regulate the passage of molecules into cells, however it is not known whether they confer antibiotic resistance, and if this potential resistance is directed at specific classes of antibiotics. This study examined whether the *Escherichia coli* K12 capsule provides resistance to the antibiotics streptomycin and tetracycline. This potential resistance was investigated by comparing a strain with a deletion of the *wza* gene, which encodes a capsular polysaccharide transport protein, to wildtype cells. The amount of capsular polysaccharides produced by the ∆wza mutant was reduced compared to wildtype cells at 21°C, but not 37°C. A broth minimal inhibitory concentration (MIC) assay determined antibiotic susceptibility, and no difference between wildtype and the ∆wza mutant was observed. Additionally, both cell types were four times more susceptible to both antibiotics at 21°C relative to 37°C. Taken together, these results indicate that the *E. coli* K12 capsule does not play a role in conferring resistance to either streptomycin or tetracycline.

Capsules have been proposed to be a source of antibiotic resistance in bacteria such as *Escherichia coli* because they can regulate the passage of various molecules through the cell envelope (1). Exopolysaccharides (EPS) are essential components of capsules, which are anchored to the cell surface of many Gram-negative bacteria including *E. coli* K12 (2). The *E. coli* *wza* gene encodes an outer membrane transport protein which exports group I capsular polysaccharides, and a knockout mutant of this gene is not known to affect other cellular processes (3). Naimi *et al.* observed that the *E. coli* K12 ∆wza mutant produced 1.25 times less capsular polysaccharides than wild-type (WT) cells (4). It is important to note that the cultures used in their experiment were grown at 37°C. However, it has been stated that capsule synthesis is diminished at 37°C relative to lower temperatures (5). Thus, *E. coli* is expected to have a smaller capsule at 37°C compared to 21°C.

Prior studies found that exposing *E. coli* to sub-lethal antibiotic concentrations resulted in an increase in both capsule thickness and antibiotic resistance (6). Nevertheless, previous studies have not produced conclusive results with regards to the role of the *E. coli* capsule in providing antibiotic resistance. Ganal *et al.* suggested that *E. coli* develops resistance to the aminoglycosidic antibiotics kanamycin and streptomycin in a capsule-dependent fashion, indicating that the capsule plays a role in conferring antibiotic resistance (1). Conversely, Drayson *et al.* proposed that *E. coli* develops resistance to these antibiotics in a manner independent of its capsule (7). Song *et al.* found that EPS in *in vitro* may attenuate the activity of certain antibiotics such as tetracycline by a mechanism that is not fully understood (8). Thus, due to the resemblance of the *E. coli* capsule to the *in vitro* EPS system it is possible that capsules provide antibiotic resistance to tetracycline through a mechanism such as attenuation (8,9). The apparent discrepancy in the literature regarding the role of capsule in mediating antibiotic resistance could be resolved if resistance is contingent upon antibiotic class.

Different classes of antibiotics have different chemical properties, which endow them with distinct mechanisms of action even if they target similar processes. Streptomycin is an inhibitor of protein synthesis as it targets the ribosome and disrupts the translation initiation and elongation steps (10). Tetracycline also inhibits protein synthesis at the translational level by irreversibly binding the 30S ribosomal subunit, thus preventing binding of tRNA to the A site of the ribosomal complex (11). Both these antibiotics must enter the cell in order to carry out their functions, and thus must be able to pass through the cell surface, which in *E. coli* K12 includes a capsule.

In this study we investigated the sensitivity of wildtype (WT) and ∆wza mutant cells to tetracycline and streptomycin. The amount of capsular polysaccharides produced by each cell type was quantified and compared at different temperatures and in different media, and the antibiotic susceptibility of both cell types was tested at various concentrations of the two antibiotics.

We hypothesized that capsular polysaccharides confer greater antibiotic resistance against tetracycline compared to streptomycin, because of the proposed attenuation of tetracycline in an *in vitro* EPS system (8). However, we found that there was no difference in resistance to either antibiotic, implying that the *E. coli* K12 capsule does not play a role in providing resistance to these two classes of antibiotics.

**MATERIALS AND METHODS**

**Bacterial strains, media preparation, and growth conditions.** *E. coli* K12 JW2047-1 (genotype: F−, Δ(araD-araB)567, ΔlacZ4787::rrnB-P1, LAM−, Δwza-760::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514) and BW25113 (genotype: F+, Δ(araD-araB)567, ΔlacZ4787::rrnB-P1, LAM−, rph-1, Δ(rhaD-rhaB)568, hsdR514) were obtained from the MICB 421 culture collection (Department of Microbiology and Immunology, University of British Columbia). JW2047-1 has a kanamycin resistance marker inserted in the *wza* locus. JW2047-1 is referred to as the ∆wza mutant and the BW25113 is denoted as wild-type (WT). All experiments were performed at either 21°C or 37°C. Liquid cultures were incubated in a water bath adjusted to the...
appropriate temperature with shaking speed of 200 rpm. Two water baths were used in order to accommodate growth at two different temperatures. Bacterial cells were grown in both Luria-Bertani (LB) broth (1.0% w/v tryptone; 0.5% w/v yeast extract, 0.5% w/v NaCl, pH 7) and Muller Hinton (MH) broth (0.2% w/v beef extract, 1.75% w/v acid digest of casein, 0.15% starch, pH 7.3, not cation-adjusted) for capsule isolation, capsule staining, and for the minimum inhibitory concentration (MIC) assay. LB broth and plates (LB with 1.5% w/v agar) were used to determine the CFU/ml to OD660 ratio for the two cell types at 21ºC and 37ºC.

**Determination of CFU/ml to OD660 ratio for each cell type at 21ºC and 37ºC.** A colony of each cell type was inoculated in 5 ml of liquid media and grown overnight at either 21ºC or 37ºC. The overnight cultures were diluted in 50 ml of fresh media the following day. The growth phases of each culture was followed by periodically taking OD660 readings using a Spectronic 20+ spectrophotometer. Once the cells reached log-phase of growth, three different OD660 readings were taken and the concentration of cells at each OD660 value was simultaneously measured by plating three different dilutions in duplicate such that they would yield 30-300 colony forming units (CFU). The final CFU/ml to OD660 ratio for each cell type was established by calculating the mean of the three CFU/ml to OD660 ratios.

**Capsule Staining.** A colony of each cell type was inoculated in 5 ml of liquid media and grown overnight at either 21ºC or 37ºC. Capsule staining of the overnight cultures was performed using the Maneval’s capsule staining method as described by Hughes and Smith with slight modifications (12). First, 10 ml of overnight culture was mixed with 10 ml of Congo Red (1% aqueous solution, Sigma Chemical Company C-6767), spread onto a glass microscope slide, and air-dried. The dried smears were then flooded in Maneval’s solution (0.047% w/v acid fuchsin, JT Baker Chemicals, A355-3; 2.8% w/v ferric chloride, Fisher Scientific 1-89; 4.8% w/v aqueous glacial acetic acid, Acros, 42322-0025; 3.6% v/v aqueous phenol solution, Invitrogen I5509-037) for five minutes. The counterstain was washed off with dH2O and the slides were air-dried before being viewed using a light microscope at 1000x magnification with oil immersion.

**Capsule extraction.** Capsule extraction was performed as described by Brimacombe et al. with slight modifications (13). A colony of each cell type was inoculated in 5 ml of liquid media and grown overnight at either 21ºC or 37ºC. The following day, OD660 readings for each culture were measured using a Spectronic 20+ spectrophotometer, while simultaneously transferring 1 ml of the same culture into a sterile microcentrifuge tube. Next, the 1 ml samples were centrifuged using an Eppendorf 5415D microcentrifuge for 2.5 minutes at 16,100 x g. The supernatants were discarded and the pellets were resuspended in 1 ml of 50 mM NaCl. The NaCl wash step was carried out four additional times before the pellets were resuspended in 1 ml of 50 mM EDTA. The samples were then incubated at 37ºC on a rotating wheel for 60 min. After incubation, the samples were pelleted at 16,100 x g and the supernatant containing cell surface polysaccharides was transferred into a fresh, sterile microcentrifuge tube.

**Capsule quantification.** Capsule quantification was performed by the phenol-sulfuric acid assay as described by Brimacombe et al. with slight modifications (13). A 1.0 mg/ml carbohydrate stock solution containing 0.05% w/v sucrose and 0.05% w/v fructose was used to prepare the standard.

**MIC Assay.** A broth microdilution minimal inhibitory concentration (MIC) assay was performed as described by Wiegand et al. with slight modifications (14). A colony of each cell type was inoculated in 5 ml of liquid media and grown overnight at either 21ºC or 37ºC. The following day, OD660 readings for each culture were measured using a Spectronic 20+ spectrophotometer. Using the CFU/ml to OD660 ratio previously established for each cell type at 21ºC and 37ºC, the overnight cultures were diluted to a concentration of 1 x 10³ CFU/ml. Stock solutions of either tetracycline or streptomycin were diluted in liquid media (LB or MH broth) to a range of doubling concentrations between 0.125-64 µg/ml. Next, 50 µl of each antibiotic solution and 50 µl of bacterial culture were transferred into a designated well of a sterile polystyrene 96-well microtiter plate (Falcon 3915) in duplicate. Since the MIC assay was performed at two different temperatures, two microtiter plates with the same antibiotic and culture dilution schemes were made. Growth and sterility controls were also included in the assay. Following overnight incubation at 21ºC or 37ºC, the microtiter plates were inspected visually for identifying the minimum concentration of antibiotics at which no growth was observed.

**RESULTS**

WT cells produce more capsular polysaccharides than Δwza mutants at 21ºC. To determine if there is a capsule biosynthesis defect in the Δwza mutant compared to WT, total EPS was quantified using a phenol-sulfuric acid method. We found that there was no significant difference in capsular polysaccharide concentration between the WT and Δwza cells grown in LB media at 37ºC (Fig. 1). In contrast, when grown in LB media at 21ºC, Δwza produced 10-fold less capsular polysaccharide (Fig. 1). Unexpectedly, capsule production of WT versus Δwza cells was approximately equal in MH media regardless of temperature (Fig. 1).

**FIG 1 Effect of growth medium and growth temperature on the recovery of capsule carbohydrate in WT and Δwza strains.**

**Capsule size varies with temperature and media.** Based on the capsule quantification results shown in Fig 3, WT *E. coli* cells were predicted to have a larger capsule at 21ºC than Δwza cells. At 37ºC, however, the white region representing capsule (Fig. 2, panels B and D) was not expected to be prominent and the two cells types were expected to appear similar after staining. Additionally, the media was not expected to affect the trends observed. In agreement with our expectations, WT cells produced larger capsule than the Δwza mutant grown at 21ºC in LB media. In addition, the capsules of WT and Δwza cells were similar at 37ºC. The same experiments done in MH media indicated that there was no difference in capsule size between WT and Δwza cells at 21ºC, a result that was not observed in LB media (Fig. 3).
The lack of a distinct capsule at 37°C, had previously investigate temperatures than wzaesman that yson wza 4) wza 4). This is important to note because wza 4). In agreement with our expectations, we found that WT cells produced 10 times more capsule than Δwza cells grown at 21°C.

Capsule stains were performed to verify a difference between WT and Δwza cells. The capsule was considerably more prominent at 21°C for WT cells grown in LB media (Fig. 2, panels A and C) than Δwza cells, which did not produce a visible capsule. This contrast between the two cell types is in agreement with the data obtained in the capsule quantification assay (Fig. 1). The lack of a distinct capsule at 37°C complements the findings of Stout and Gottesman that capsule synthesis decreases at temperatures reaching 37°C (5).

The MIC data for both tetracycline and streptomycin indicate that capsule does not play a role in antibiotic resistance (Fig. 4 and 5). This is in agreement with the findings of Drayson et al., which state that capsular synthesis and antibiotic resistance are independent of one another (7). We then speculated that using different media for capsule quantification and the MIC assay may have played a role in the observed results. Bearing in mind Seo and Matthews’ findings regarding the influence of media on capsule synthesis, we chose to investigate media type as an additional variable that may affect capsule synthesis (15).

**DISCUSSION**

WT and Δwza cells grown at 37°C in LB media produced approximately the same amount of capsule (Fig. 1). This was consistent with prior observations that capsule synthesis is lower at 37°C than lower temperatures (5). This is important to note because Naimi et al. had previously investigated the antibiotic susceptibility of WT and Δwza cells to streptomycin at 37°C, but did not observe a relationship between capsule size and antibiotic resistance (4). In hindsight, this was consistent with a decrease in capsule synthesis at 37°C, which would diminish differences in capsule size between WT and Δwza cells (5). We thus chose to investigate growth at not only 37°C, but also at a lower temperature of 21°C. WT E. coli cells were predicted to possess a thicker capsule at 21°C because knocking out wza, encoding the group I capsular polysaccharide transporter Wza, would prevent export of capsular polysaccharides and thus reduce capsule size (3). In agreement with our expectations, we found that WT cells produced 10 times more capsule than Δwza cells grown at 21°C.

**E. coli K12 capsule does alter antibiotic resistance.** We hypothesized that WT cells would be more resistant to tetracycline than Δwza cells. In addition, susceptibility to tetracycline was predicted to be greater at 37°C than at 21°C. Susceptibility to streptomycin, however, was not expected to differ between the two cell types or across temperatures. We found that the WT and the Δwza cells were equally sensitive to streptomycin and tetracycline (Fig. 2). In contrast between the two cell types or across media for capsule quantification and the MIC assay may have played a role in the observed results.

FIG 2 The effect of growth temperature on capsule thickness between WT and Δwza cells when grown in LB broth. (A) WT cells grown at 21°C; (B) WT cells grown at 37°C; (C) Δwza cells grown at 21°C; (D) Δwza cells grown at 37°C. Capsules of all cells were stained using Maneval’s staining method and visualized at 1000X magnification. The white region represent capsule, while gray regions represent the cell body.

FIG 3 The effect of capsule thickness between WT and Δwza cells when grown in MH broth. (A) WT cells grown at 21°C; (B) WT cells grown at 37°C; (C) Δwza cells grown at 21°C; (D) Δwza cells grown at 37°C. Capsules of all cells were stained using Maneval’s staining method and visualized at 1000X magnification. The white region represent capsule, while gray regions represent the cell body.
An interesting commonality of the two MIC assays was that both cell types were four times more susceptible to tetracycline and streptomycin at a lower temperature of 21°C relative to 37°C. This increased susceptibility at lower temperatures has been previously assessed in *Pseudomonas syringae* (16). Kumar *et al.* found that *P. syringae* was more susceptible to polymyxin B at 4°C when compared to a higher temperature of 22°C (16). This increase in resistance was attributed to a decrease in fluidity of membrane lipopolysaccharides (16). Although the experiment performed by Kumar *et al.* utilized a different bacterial system and antibiotic, it is possible that a similar mechanism may mediate the increased susceptibility of *E. coli* K12 to streptomycin and tetracycline at 21°C relative to a higher temperature of 37°C. However, a comparison of 4°C and 22°C to 21°C and 37°C, respectively, is not entirely justified and will likely require further investigation in the future.

The observation that both cell types were four times more resistant to streptomycin when incubated in LB media as opposed to MH media regardless of the temperature is also noteworthy (Fig. 4). This may be attributable to the findings of Beggs *et al.*, which state that increasing the ionic strength of a solution via increasing salt content results in inhibition of aminoglycoside action (17). Therefore a potentially greater ionic strength in the LB media as compared to MH media may explain the higher MIC values observed for *E. coli* K12 to the aminoglycoside streptomycin (17). In contrast, resistance to tetracycline was found to be independent of the media in which cells were incubated. Since WT cells grown in LB media were shown to possess a thicker capsule than those in MH media, a lack of change in tetracycline susceptibility further supports that resistance to it is not mediated by capsular polysaccharides.

It is important to note that although the capsular polysaccharide content was quantified using exponential phase cells, the microscopic images presented in Figure 1 and 2 were of stationary phase cells. An experiment conducted by Wooster *et al.*, however, showed that the media of stationary phase *E. coli* K1 contained approximately 10 times more capsular polysaccharide than the media of exponential phase cells (18). This suggests that stationary phase *E. coli* cells continue to produce capsule while also shedding old capsule into the media (18). Thus, although the microscopic data and the capsular quantification data are correlated, the stationary phase cells in the microscopic images of Figure 1 and 2 may in fact have greater capsular polysaccharide concentrations than those indicated in the capsule quantification assay. The trends, however, are still maintained. The argument could also be reversed by arguing that the stationary phase cells have more shedding so that less capsule is retained on the cells so the capsule of stationary phase cells might look thinner than the capsule of cells in exponential growth. The observation is also a bit disingenuous because the shed capsule would accumulate over the growth period so the media of the cultures that grew longer (the cells in stationary phase) should have more shed capsule.

Additionally, it is important to further consider the large difference in the thickness of the capsule synthesized in LB media versus MH media. The lack of a difference in capsule content between the cell types or across temperatures in MH media may be attributed to the contents of the media itself. In particular, glucose, potassium, phosphate, magnesium, and certain trace elements are necessary for CPS production; in addition, increasing concentration of carbon and nitrogen sources in growth media have been shown to increase capsule production (15). Although the aforementioned study was conducted in *E. coli* O157:H7, the relatively similar capsular gene repertoire of various *E. coli* strains suggests that it may be applicable to the related *E. coli* K12 used in this experiment (15,19). More specifically, cells grown in MH may produce less capsule than in LB due to inherent limitations in specific nutrient sources.

It should be of note that tetracycline was previously shown to be attenuated by an extracellular polysaccharide matrix consisting of not only polysaccharides but also proteins (8). Song *et al.* suspected that the interactions between tetracycline and

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**FIG 4** Effect of growth medium and growth temperature on the MIC of streptomycin for WT and Δwza strains.

**FIG 5** Effect of growth medium and growth temperature on the MIC of tetracycline for WT and Δwza strains.
the matrix might have been electrostatic antibiotic-protein interactions and not tetracycline-polysaccharide interactions. Thus, the identical MIC values obtained for the WT and Δωza cells may be attributed to a similar capsular protein content between the WT and mutant cell types.

Secondly, it is important to address the issue of cross-resistance between aminoglycosides. As Naimi et al. were not able to identify cross-resistance to streptomycin due to the kanamycin resistance gene present in Δωza cells, it was not considered further in this study. However, due to kanamycin and streptomycin both belonging to the aminoglycoside class of antibiotics and thus exhibiting similar chemical properties, there is still a possibility that the kanamycin resistance gene provided cross-resistance to streptomycin. If this cross-resistance was provided, it could have potentially negated any differences in MIC values that may have been observed if capsule size had been the only variable. A repetition of this study but with the kanamycin resistance gene deleted would serve to better elucidate the effect of any cross-resistance mediated by the kanamycin resistance gene.

In conclusion, the results obtained indicate that E. coli K12 capsule does not provide measurable resistance to streptomycin and tetracycline, representing the aminoglycoside and tetracycline classes of antibiotics, respectively.

FUTURE DIRECTIONS

A more thorough examination of the relationship between capsule size and antibiotic resistance should be conducted by utilizing a broader range of antibiotics in order to determine whether the trend observed in this study applies more generally to other antibiotic classes as well. Adaptation of the methods utilized by Park et al., which involved incubating E. coli B23 cultures with purified EPS to determine potential kanamycin-EPS interactions, can be used to assess the interaction of a range of antibiotics with crude and purified capsules (20). This may better assist in uncovering potential interactions. Based on our findings, we also propose that future experiments assessing various roles of the E. coli capsule be conducted at the lower temperature of 21°C versus 37°C. It was apparent from our results that WT E. coli produce thicker capsules at 21°C. Furthermore, future experiments should be conducted with E. coli strains deficient in capsular proteins to test the validity of speculations made in this paper as well as by Song et al. stating that protein-tetracycline interactions may be responsible for tetracycline attenuation (8). In this way, comparison of tetracycline resistance to WT cells possessing all capsular proteins versus a mutant lacking some or all capsular proteins would elucidate whether it is in fact the residual protein components of bacterial capsules that play a role in antibiotic resistance.

Moreover, the findings of Seo and Matthews and Torres-Cabassa et al. suggest that media composition influences capsule synthesis (15, 21). Hence, future experiments could quantify and compare capsular polysaccharide content as a function of media composition by altering media properties such as salt concentration, pH and selection of divalent cations. Finally, in order to better correlate quantitative capsule polysaccharide data to capsule strains, future studies should look into protocols that allow for the use of cells in identical phases of growth.

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