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# Increased Antibiotic Resistance Post-exposure to Sub-inhibitory Concentrations is Independent of Capsular Polysaccharide Production in *Escherichia coli*

**Robyn Drayson, Trevor Leggat and Melissa Wood**
*Department of Microbiology & Immunology, University of British Columbia*

**Previous experiments have shown that pretreatment of *Escherichia coli* with sub-lethal doses of antibiotic resulted in a measurable increase in capsule production and resistance upon secondary exposure. Whether this increase in capsule was directly implicated in antibiotic resistance had been investigated by numerous papers, but conclusive evidence supporting a link has yet to be found. In this study the use of *E. coli lon* and *rcsB* genetic mutants, which over and under produce the capsular polysaccharide colanic acid respectively were utilized to mimic capsule overproduction and deficiency; colanic acid being a constituent of capsule. We investigated the role of capsular polysaccharide production in antibiotic resistance by measuring changes in turbidity as a measure of growth. Despite the *lon* strain having displayed both a microscopically and macroscopically observable increase in capsule production, there was no discernible difference in turbidity increase between it and the down-regulated strain in the presence of varying concentrations of kanamycin or streptomycin. Our data support the conclusion that antibiotic pre-treatment independently induces both increased antibiotic resistance and capsular polysaccharide production.**

The prevalence of antibiotic resistant pathogens has become a serious health concern, rendering the study of resistance mechanisms critical (13). Previous studies have shown that when *Escherichia coli* is exposed to sub-inhibitory levels of antibiotic not only is there increased resistance to the specific antibiotic used, resistance to other structurally and functionally unrelated antibiotics is also observed which suggests a broad resistance mechanism (2). One of the proposed explanations for the non-specific resistance comes from the observation that exposure to sub-inhibitory antibiotic concentrations causes increased production of capsular polysaccharide in *E. coli* (4, 7, 8, 10, 12).

Capsule is a term encompassing both membrane anchored and exopolysaccharides and is implicated in the access of certain molecules to the bacterial envelope, the promotion of adherence to objects, the formation of biofilms and prevention of desiccation (9). Classification of capsular polysaccharides is based on properties dependent on the linkage between the polysaccharide and outer membrane lipids, but the exact linkages of many have not been elucidated (16). Capsular polysaccharides are serologically divided into two main groups, and while colanic acid shares many characteristics with group I capsular polysaccharides it is not classified in this way (16). Despite colanic acid not being classified as either a type I or II capsular polysaccharide, it is still considered part of bacterial capsule and altering its production is a valid means of studying the effect of capsule on antibiotic sensitivity (9, 16).

While increases in capsule production and antibiotic resistance after exposure to sub-inhibitory concentrations have been observed, whether this resistance mechanism is dependent or independent of capsule production has yet to be demonstrated (4, 7, 8, 10, 12). Numerous experiments have used genetic mutants in an attempt to determine the role of capsular polysaccharide production in this resistance mechanism (4, 6, 8, 12). *E. coli* strains with mutations in *cpsG* and *cpsB*, genes essential for capsular colanic acid biosynthesis, did not negatively affect growth upon secondary antibiotic treatment (4, 8). An *E. coli* strain with a mutation in *wza,* a gene involved in the translocation of type I polysaccharides across the membrane, was also used but again did not increase antibiotic sensitivity (12). Finally, Fowler *et al.* employed strains having deletions in Rcs regulatory network genes but did not obtain conclusive results (6).

The Rcs regulatory network was initially discovered because of its role in capsule production, but has since been observed to be involved in regulation of many other processes (11, 14). Changes in osmolarity, temperature, envelope stress and overproduction of specific membrane components results in activation of the membrane bound sensor kinase RcsC, which phosphorylates RcsB, both of which are constitutively expressed (14). The activated form of RcsB forms a heterodimer with RcsA, which activates the

transcription of over 150 genes, including those involved in the activation of membrane biofilm formation, motility, antibiotic resistance, cell division, and the synthesis of *cps* operon encoded colanic acid (14, 16). RcsA is produced at low rates in addition to being acted upon by the Lon protease, which maintains RcsA at transient levels regardless of environmental conditions (15). Therefore strains with mutations in *lon (*JW0429-1) and *rscB* (JW2205-2) are expected to have upregulated and downregulated transcription of genes involved in the synthesis of capsule, respectively.

In this study we used the *lon* and *rcsB* genetic mutants as a tool to mimic capsule overproduction and deficiency to investigate the role of capsule in antibiotic resistance. The use of genetic mutants allowed us to remove the pretreatment step, minimizing any other cellular modifications that may occur in response to antibiotic treatment. We hypothesized that if capsule was involved in protection against antibiotics (particularly aminoglycosides) the up-regulated mutant should have increased resistance and the down-regulated mutant should be more susceptible. Conversely, if there is no discernible difference in antibiotic sensitivity between these strains it would provide support for the conclusion that up-regulation of capsule is independent of observed increases in antibiotic resistance against aminoglycosides.

This is an important area of research as it allows us to improve our understanding of antibiotic resistance mechanisms and potentially learn new ways to counter the ever-growing threat of antibiotic resistant infectious bacteria. We attempted to test the involvement of capsule in antibiotic resistance by use of the *Escherichia coli* genetic mutants DLW11w1 (a *lon* mutant) and DLW11w2 (an *rcsB* mutant), which were intended to over and under produce capsule respectively.

## MATERIALS AND METHODS

**Bacterial strains, media preparation and growth conditions.** *E. coli* B23, BW25113, JW0429-1 and JW2205-2 strains were obtained from the MICB 421 laboratory stock from the Department of Microbiology and Immunology at the University of British Columbia, Vancouver B.C. (Table 1). JW0429-1 and JW2205-2 strains were Keio strains (1) originally provided by the Coli Genetic Stock Center, Yale University. JW0429-1 and JW2205-2 are derivatives of the parental BW25113 strains, which overproduce and underproduce capsule respectively (Table 1). The DLW11w1 strain is a derivative of JW0429-1 with the kanamycin resistance gene removed. The DLW11w2 strain was also prepared from the parental JW2205-2 strain with the kanamycin resistance gene removed. All experiments were performed at room temperature (approximately 21°C) unless otherwise specified. Dehydrated Mueller-Hinton (MH) broth (Difco #0757-01-4) was dissolved in distilled water according to the manufacturer's instructions and autoclaved. Liquid media cultures were grown in a shaking water bath. The MH plates used throughout the study were prepared by adding 1.5% w/v agar (Invitrogen Select #30391-023) to MH broth. All media was stored at room temperature prior to use. Bacterial cells were grown in Luria-Bertani (LB) broth (0.5% w/v Bacto Yeast Extract, 1% w/v Bacto Tryptone, 0.5% w/v NaCl, 2% agar, pH 7) for the deletion of kanamycin resistance and grown on LB plates with 1.5% w/v agar. In addition M9 minimal media (8.5 mM NaCl, 49 mM $Na_2HPO_4$, 22 mM $KH_2PO_4$, 18 mM $NH_4Cl$, 0.80 mM $MgSO_4 \cdot 7H_2O$ supplemented with 0.4% w/v glycerol as carbon source) was utilized as part of the determination of optimal growth conditions testing.

**Deletion of kanamycin resistance in *E. coli* JW0429-1 and JW2205-2.** In order to test sensitivity to kanamycin, the kanamycin resistance gene was removed from the JW0429-1 and JW2205-2 strains. The pCP20 plasmid obtained from the MICB 421 laboratory stock (ts-rep, [c1857](λ)(ts), bla(ApR), cat, FLP(II), ampicillin-resistant, chloramphenicol-resistant) was used to delete the kanamycin resistance gene. The JW0429-1 and JW2205-2 strains were transformed with the pCP20 plasmid, which allowed for the removal of kanamycin resistance according to the protocol outlined in Cho *et al.* (4). Cells were electroporated in the presence of the pCP20 plasmid, plated on LB-ampicillin plates (1.5% agar, 100 µg/ml ampicillin) and incubated overnight at 37°C. Ampicillin resistant cells were chosen, streaked on LB plates and incubated overnight at 42°C so that transcription of the pCP20 FLP(II) was activated, removing kanamycin resistance, in addition to the plasmid replication being deactivated. Cells were then plated on LB-ampicillin and LB-kanamycin (100 µg/ml kanamycin) plates and colonies with

**TABLE 1.** Notable mutations of *E. coli* strains used, source, and resulting phenotype of interest.

| Strain | Phenotype | Noteworthy Mutations | Source |
|---|---|---|---|
| B23 | Wild-type | N/A | MICB 421 laboratory stock, Department of Microbiology and Immunology UBC |
| BW25113 | Parental of JW0429-1, JW2205-2 and their derivatives | F-, *Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), LAM-, rph-1, Δ(rhaD-rhaB)568, hsdR514* | Coli Genetic Stock Centre |
| JW0429-1 | Mucoid | F-, *Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), Δlon-725::kan, LAM-, rph-1, Δ(rhaD-rhaB)568, hsdR514* | Coli Genetic Stock Centre |
| DLW11w1 | Mucoid, kanamycin resistance removed derivative of JW0429-1 | F-, *Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), Δlon-725, LAM-, rph-1, Δ(rhaD-rhaB)568, hsdR514, with pCP20 plasmid* | This study |
| JW2205-2 | Capsule deficient | F-, *Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), LAM-, ΔrcsB770::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514* | Coli Genetic Stock Centre |
| DLW11w2 | Down-regulated capsule, kanamycin resistance removed derivative of JW2205-2 | F-, *Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), LAM-, ΔrcsB770, rph-1, Δ(rhaD-rhaB)568, hsdR514, with pCP20 plasmid* | This study |

sensitivity to kanamycin were chosen.

**Determination of optimal growth conditions for capsule production.** Previous papers had shown variance in temperature and media used for induced antibiotic resistance tests (4, 7, 8, 10, 12). To determine the media/temperature combination that allowed for the greatest difference in capsule production *E. coli* JW0429-1, JW2205-2, B23 and BW25113 were streaked on Mueller Hinton (1.5% agar) and M9 minimal media (1.5% agar) plates, which were incubated at either 42°C or room temperature. Room temperature plates were left for 3 days while 42°C plates were left overnight. The mucoidy of each strain was noted.

**Staining of Capsule.** Strains were streaked on MH plates and grown at room temperature for 3 days prior to being stained. Staining of capsule was completed according to the staining technique outlined by Chiang *et al.* (3). A 1% Congo red (Sigma #6767) solution was prepared. The second staining solution, Maneval's stain, was prepared by combining 15 ml of 5% aqueous phenol solution (Analar BDH Chemicals #B10188), 4.5 ml of 20% aqueous glacial acetic acid, 2 ml of 30% aqueous ferric chloride (Fisher #7705-08-0), and 0.75 ml of aqueous solution of acid fuchsin (Baker #232-2119). Stains were stored at room temperature prior to use.

To stain capsule for visualization, a drop of a 1% Congo red solution was placed at the end of each glass slide and mixed with a loopful of culture. A second clean slide was then placed at an angle and backed-into this mixture allowing for the generation of a thin smear. The sample was left to air dry. After the sample had air-dried, a few drops of Maneval's stain was spread using the same method as the Congo red stain and left to stand for 60 seconds. It was then rinsed with distilled water and blotted dry with paper towel. The samples were then visualized at 1000X magnification under oil immersion using a Nikkon Eclipse TE2000-U microscope. Photographs were then taken with a Qimaging Micropublisher 5.0 RTV Nikkon T-BDCA digital camera set to 1X magnification hooked up to the microscope in the Gaynor lab.

**Determination of $OD_{595}$ to CFU/ml ratio for individual strains.** Overnight cultures of *E. coli* DLW11w1, DLW11w2, B23 and BW25113 were grown in a shaking water bath at room temperature. The following day $OD_{595}$ readings were taken periodically to ensure cells were in log phase of growth. Cultures were then diluted accordingly to yield final plated dilutions of 30-300 colony forming units (CFU) when plated in duplicate.

**Antibiotic growth assay.** In accordance with Fowler *et al.* we performed an antibiotic growth assay as opposed to a minimum inhibitory concentration (MIC) assay in order for comparability of results (6). MH media was inoculated with overnight cultures of DLW11w1, DLW11w2, B23 and BW25113 to yield bacterial concentrations of 4 - 8 $x10^5$ CFUs/ml. This was calculated using the previously determined $OD_{595}$ to CFU/ml ratio for individual strains. Antibiotic concentrations of 8, 4, 2, 1, 0.5 and 0 µg/ml were prepared for both kanamycin and streptomycin using distilled water. Two 96-well plates (Corning #9667) were then set up in triplicate for each of the four strains with 1 row serving as a blank/sterility control (200 µl of MH media), the remaining 7 rows were set-up to each contain a single antibiotic concentration (50 µl MH, 50 µl antibiotic, 100 µl culture in each well). Final plated concentrations of the antibiotic and bacterial cells were therefore a quarter of the stock. Plates were left to incubate at room temperature for 24 hours. Turbidity readings were taken with a BioRad Model 3550 Microplate reader at 595 nm both at time zero and after the 24 hour incubation. Change in turbidity at 595 nm was determined by subtracting the initial day zero turbidity from the final turbidity value at 24 hours.

## RESULTS

**Removal of kanamycin resistance cassette from *E. coli* JW0429-1 and JW2205-2.** *E. coli* JW0429-1 and JW2205-2 strains contained a kanamycin resistance cassette which needed to be removed in order to test strain sensitivity to kanamycin. A number of other studies had previously looked at sensitivity to kanamycin and Fowler *et al.* used streptomycin; hence we decided to investigate sensitivity to both streptomycin and kanamycin (4, 6, 7, 8, 10, 12). JW transformation with the pCP20 plasmid successfully removed kanamycin resistance while maintaining phenotypic differences (Figures 1 and 2), however the cells remained ampicillin resistant, suggesting the pCP20 plasmid remained within these strains, now DLW11w1 and DLW11w2 respectively (Table 1). It was assumed the presence of the pCP20 plasmid did not contribute to the observed results in subsequent tests since it was present within both experimental strains. Ampicillin resistance was not tested in this study so the strains were used despite retention of the plasmid.

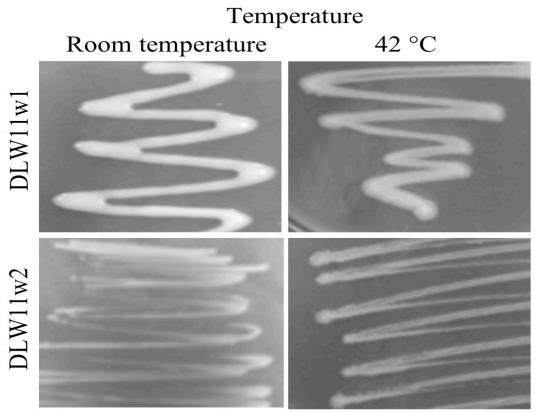


**FIG. 1. Growth on Mueller Hinton agar plates at room temperature demonstrated the greatest difference in capsule production.** The DLW11w1 and the DLW11w2 strains were compared for colony characteristics and mucoidy after growth at room temperature and 42°C. Visible mucoidy was used as a measure of capsule production.

**Growth on MH at room temperature resulted in the greatest macroscopic phenotypic variance.** When grown on MH at room temperature the strains were most visibly different in their capsule production, as determined by the apparent mucoidy of colonies (Figure 1). When the different strains were grown on M9 minimal media at room temperature and 42°C there was a much smaller observable difference in the degree of mucoidy (data not shown). All later tests were performed using MH at room temperature as this was determined to be the optimal growth condition.

**Capsule staining showed massive capsules in DLW11w1.** Staining of the DLW11w1 strain revealed the presence of massive capsules. The other 3 strains were fairly similar to each other as determined by microscopy (Figure 2). No comparison of capsule was performed on cells after exposure to antibiotic.

**Excess capsule does not clearly show a protective function.** Both kanamycin and streptomycin exposures

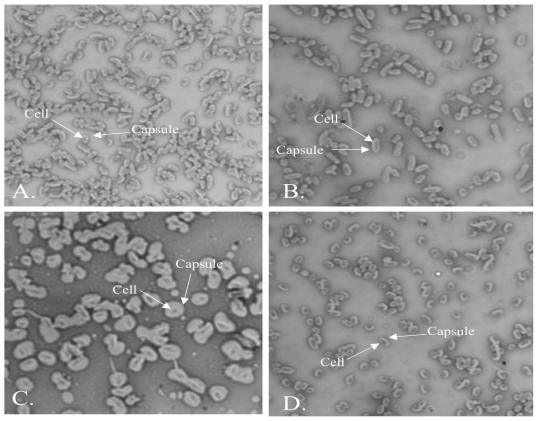


**FIG. 2. The effect of genetic mutations on bacterial capsule production.** *E. coli* strains B23 (A), BW25113 (B), DLW11w1 (C), and DLW11w2 (D) stained for visualization of capsule using a negative staining method utilizing a two step staining procedure of Congo red followed by Maneval's staining solution, observed under 1000X magnification. White halos are capsule while dark centers are the cell body.

displayed similar trends in growth where none of the strains were affected by the addition of either antibiotic at concentrations equal to or less than 0.25 µg/ml, but almost entirely inhibited by 2.0 µg/ml (Figures 3 and 4). Despite the extreme over production of capsule displayed by strain DWL11w1, the BW25113 strain displayed approximately equal amounts of growth at the different antibiotic concentrations tested. Strain DWL11w2 in the presence of streptomycin displayed growth that was consistently poorer, however its relative growth was not any worse (Figure 4). The term relative growth is used here to mean the ratio of growth at the concentration of interest compared to the growth under the condition of no added antibiotic.

All four strains are fairly similar at less than 0.25 ug/ml relative to their individual maximal changes in growth in the presence of both streptomycin and kanamycin (Figure 3 and 4). B23, changing approximately 0.19 $OD_{595nm}$, grew best at these apparently non-inhibitory concentrations but conversely was most impacted at the higher antibiotic concentrations. In the presence of kanamycin all other strains changed approximately 0.11 $OD_{595nm}$ units across concentrations of 0-0.25 ug/ml, where any differences are assumed to be minor. In the presence of streptomycin $OD_{595nm}$ differences between DLW11w1 and DLW11w2 were more substantial with DLW11w1's turbity increasing approximately 0.04 $OD_{595nm}$ units more than DLW11w2.

## DISCUSSION

In this study we were unable to show a conclusive influence of capsule on aminoglycosidic antibiotic resistance. Despite DLW11w1 producing massive capsules compared to the other tested strains it did not show increased resistance; in general the strains had approximately equal tolerance. This could be the product of a variety of issues, such as uncertainties with our experimental strains or the environmental conditions they were exposed to. However, it may simply be an indication that capsule (colanic acid specifically) does not play a role in resistance to aminoglycosidic antibiotics.

Numerous studies have found a correlation between increased antibiotic resistance and capsule production, although they have not directly been linked (4, 7, 8, 10, 12). Capsule levels had been chemically altered and numerous genetic mutants have been used in an attempt to elucidate a direct link without success (4, 7, 8, 10, 12). In particular, in the study by Fowler *et al.* Strains JW0429-1 and JW2205-2 (Table 1) were exposed to aminoglycosidic antibiotics following the pretreatment procedure described by many earlier experiments (3, 6, 7, 8, 10, 12). Not only was there no discernible difference in antibiotic resistance upon secondary antibiotic exposure between strains, there was also no difference in resistance between those pre-exposed to sub-lethal concentrations of antibiotic and those not, failing to reproduce previous findings by multiple authors (3, 6, 7, 8, 10, 12). We postulated that the inconclusive results obtained by Fowler *et al.* were due to the simultaneous use of both pretreatment and genetic mutants (6). Pretreatment may have induced general stress responses, which may have contributed to poor results (2). Not including the pretreatment allowed for the investigation of the effect of the genetic mutations without this.

However, there was no significant difference in antibiotic resistance observed among our four strains in the absence of pretreatment. Strain DLW11w1 clearly displayed an enlarged capsule compared to the other strains (Figure 2), but it did not show a significant improvement in antibiotic resistance compared to BW25113. Furthermore, strain DLW11w2 did not perform, in a relative sense, worse than any of the other strains in terms of resistance to either antibiotic. Strain DLW11w2 was actually closest to its maximum growth at concentrations such as 0.5µg/mL, where partial inhibition of all strains was observed.

There were, however, uncertainties that may complicate the interpretation of the results. The lower changes in optical density values obtained for strain DLW11w2 might be the product of its *rcsB* mutation, which has also been shown to regulate genes involved in cell division (12, 15). Even in the absence of antibiotic, strain DLW11w2 displayed roughly 70% of the growth displayed by the other strains. As a result, the relatively high growth of strain DLW11w2 at partially-inhibitory antibiotic concentrations may simply be the product of this strain exhibiting a lower

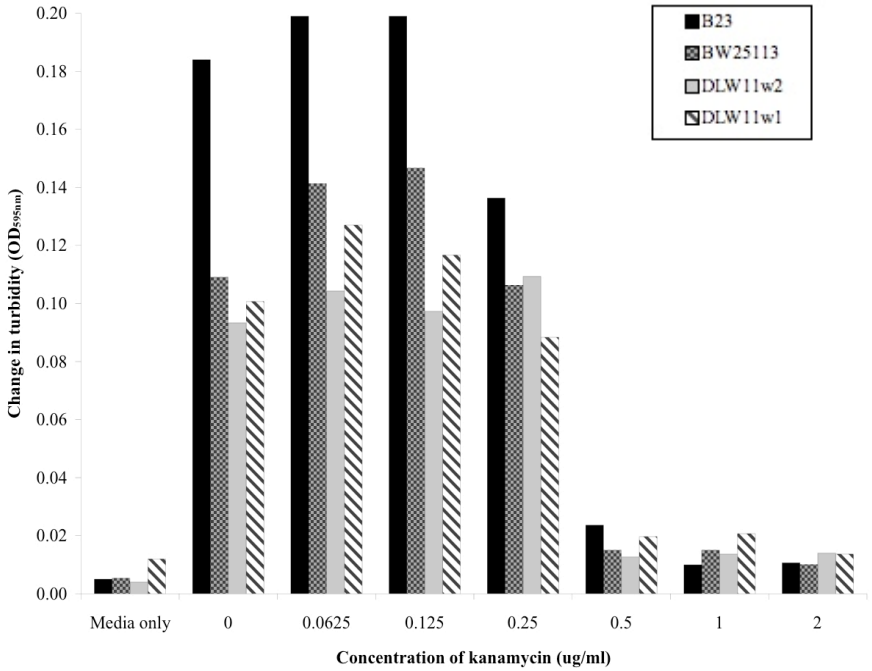


**FIG. 3. The effect of kanamycin on turbidity, as a measure of growth.** Change in turbidity at 595 nm was determined by subtracting the initial day zero turbidity from the final turbidity value at 24 hours.

level of maximum growth rather than this strain being more tolerant to antibiotics. The growth rate of strain DLW11w2 was observed to be only slightly slower than the other strains (data not shown). As a consequence strain DLW11w2 theoretically could have reached similar cell densities, albeit slightly lower, to those obtained by the other strains within the same time frame and same conditions. Furthermore, upon microscopic inspection of capsule quantity strain DLW11w2 did not show any clear decrease in capsule compared to the BW25113 or B23 strains (Figure 2). Thus, based on the hypothesis that capsule moderates resistance we would not expect a significant difference between these strains. In addition to uncertainties with the *rcsB* mutations, Lon does not act solely upon RcsA, thus the genetic alteration present in strain DLW11w1 could have impacted the bacterial cells yielding results that do not reflect alterations due to capsule alone. For example, one major function of Lon in wild type *E. coli* cells is the degradation of misfolded proteins (5). During the initial stages of streptomycin activity the antibiotic molecules interact with chain-elongating ribosomes, causing an increase in misreading of mRNA and misfolded proteins (5). These misfolded proteins can form aggregates in the membrane creating pores, which could facilitate antibiotic uptake (5). Although strain DLW11w1 demonstrated equivalent growth to strain BW25113 when no antibiotic was present, we cannot be sure if these pores in the membrane counteract any resistance the capsule itself may have conferred. Thus, the nature of the mutants used limited our ability to attribute our results solely to the capsule. Finally, while minor because of its prevalence within all experimental strains, since the pCP20 encodes ampicillin and chloramphenicol resistance there remains the risk that these strains could have some potential for cross resistance in comparison to the parental strain.

Furthermore the B23 strain, despite having visibly similar quantities of capsule as strains BW25113 and DLW11w2, was more susceptible to both antibiotics, especially kanamycin, which fails to support our original hypothesis. Perhaps differences in capsule composition contributed to this effect but based on our findings it seems more likely that it was the product of some other genetic difference between the strains. This suggests that capsule is only a minor mediator of antibiotic resistance, if at all.

Genetic uncertainties are not the only potential source of confounds in our study. Prior to performing the growth experiments we tested MH and M9 media at different temperatures (Room temperature and 42°C) to determine if environmental growth conditions could be used to amplify capsule production differences between strains. Whitfield *et al.* reported that colanic acid, as a part of capsule, production in *E. coli* increased at lower temperatures (17). The results of our growth tests support this conclusion as growth on MH at room

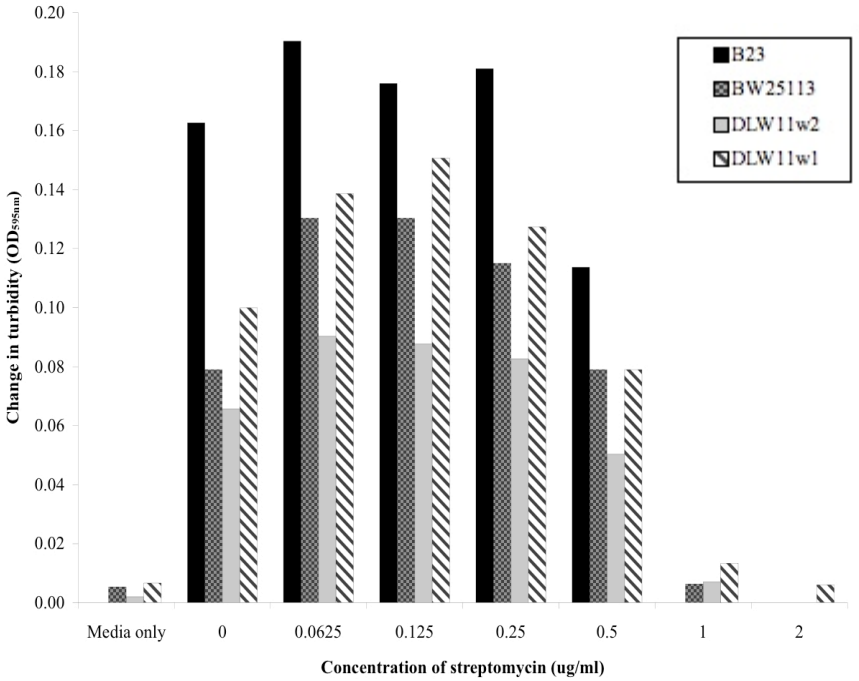


**FIG. 4. The effect of streptomycin on turbidity, as a measure of growth.** Change in turbidity at 595 nm was determined by subtracting the initial day zero turbidity from the final turbidity value at 24 hours.

temperature resulted in the greatest visible difference in mucoidy between the capsule up-regulated and down-regulated strains, as observed in Figure 1 (17). The use of these environmental conditions for this study limited our ability to compare our findings to those of Fowler *et al.* and others as they have used much higher growth temperatures (6). The change in temperature could potentially affect systems other than those involved in capsule production as temperature is known to have an effect on a variety of cell components such as membrane fluidity, types of protein synthesized and speed of response (16). Additionally, type II capsule components are thermally regulated, so the composition of the capsule may be different (16). Thus the observation that capsule production had no effect on growth rates could have arisen as a by-product of other temperature affected systems.

Likewise, there is also the possibility that capsule is still involved in some forms of antibiotic resistance but that the antibiotic and manipulated capsule component combination used in this study were not complementary. We only investigated aminoglycosides, it is possible that aminoglycosides are not impacted by capsule but other antibiotic classes are and the cells produce capsule non-specifically in response to any antibiotic threat. Similarly we altered capsule quantity by only modifying colanic acid, which is a component of capsule. Perhaps a different or additional constituent(s) of capsule is upregulated during the bacterial response to antibiotics and that colanic acid alone does not impact aminoglycosides.

In conclusion the assays used in this study were unable to detect any influence of capsule on antibiotic resistance, despite any complications that may have arisen from the genetic mutations within the strains. Our findings combined with previous studies having failed to find a link between capsule production and antibiotic resistance suggests the antibiotic resistance observed after treatment with sub-inhibitory concentrations is independent of increased capsule synthesis. We initially hypothesized capsule synthesis was induced after pretreatment with sub-inhibitory levels of antibiotic and this conferred the increased antibiotic resistance. However, the increased antibiotic resistance and capsule production appear be independent of one another. We tested our initial hypothesis using strains that over and under produced capsule in the absence of an antibiotic pretreatment. No effect of capsule production on antibiotic resistance was seen hence our data fail to support our initial hypothesis. Assuming that the above issues did not impact our results, we found no compelling evidence that capsule plays a role in antibiotic resistance. The data support the conclusion that the increased antibiotic resistance and capsule production are independent of each other.

## FUTURE EXPERIMENTS

We have shown that aminoglycosidic antibiotic resistance and capsule production are independent of one another, yet there must still be a reason for both the increased capsule production and antibiotic resistance. Due to the number of studies that have failed to support the hypothesis that increased capsule production is responsible for the observed increased antibiotic resistance post pretreatment it seems unlikely that capsule mediates the increased resistance. As such future experiments should focus on attempting to determine the mechanism of antibiotic resistance and why capsule is also up regulated in the response to antibiotic pre-treatment.

To provide further support for our conclusions this experiment could be repeated using a minimum inhibitory concentration (MIC) assay. If the results obtained in this study are repeatable, using another assay such as an MIC would further increase confidence in our results. Repeating this experiment would either result in one of two possible outcomes. If in fact increased capsule production and increased antibiotic resistance are linked, we would expect an observed increase in antibiotic resistance as the amount of capsule present increases and a decrease in antibiotic resistance in a strain that under produces capsule. Thus we would expect a lower MIC for the down regulated strain (DLW11w2) and a higher MIC for the up regulated strain (DLW11w1). The alternate outcome is that if the two are indeed independent, as our results suggest, we would expect no significant change in antibiotic resistance with changes in capsule production. In addition these tests could be performed using different antibiotics, in particular antibiotics belonging to classes other than aminoglycosides. Likewise, previously tested mutants, such as *wza, cpsG*, and *cpsB* can be used test the influence of alternate capsule components.

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