Journal of Experimental Microbiology and Immunology (JEMI)

Deletion of the *Escherichia coli* K30 Group I Capsule Biosynthesis Genes *wza, wzb* and *wzc* Confers Capsule-Independent Resistance to Macrolide Antibiotics

Sandra Botros, Devon Mitchell, Clara Van Ommen
Department of Microbiology and Immunology, University of British Columbia

The *Escherichia coli* capsule functions to protect bacterial cells from desiccation and environmental stresses. The *E. coli* group I capsule is polymerized and transported to the surface of the cells through the action of the *wza, wzb* and *wzc* gene products. It is thought that the presence of a capsule may confer a level of intrinsic antibiotic resistance. Previous work exploring the role of capsule in antibiotic resistance showed inconsistent results between different studies, and that the role of capsule in antibiotic resistance may be dependent on antibiotic class. In this study we sought to examine the role of the *E. coli* K30 group I capsule in antibiotic resistance across ten different antibiotic classes. We examined the *E. coli* K30 strain CWG655Δ[*wza-wzb-wzc*$_{K30}$] that has a chromosomal deletion of three key capsule biosynthesis genes (*wza, wzb* and *wzc)* and its isogenic parental strain E69. We quantified the capsule production of both strains and compared the susceptibility of the strains to ten different antibiotics. In doing so, we identified macrolide antibiotics as a class of interest and further examined the susceptibility of the strains to additional macrolides and a ketolide. We observed that CWG655Δ[*wza-wzb-wzc*$_{K30}$] exhibited diminished production of capsular polysaccharides compared to E69 at 21°C, but that both strains produced comparably low amounts of capsule at 37°C. Contrary to past work on other antibiotic classes, we observed that CWG655Δ[*wza-wzb-wzc*$_{K30}$] was more resistant to macrolide antibiotics, but not ketolides, when compared to E69 at both 21°C and 37°C. From this study, we conclude that a deletion of the capsule biosynthesis genes *wza, wzb* and *wzc* confers resistance to the macrolide family of antibiotics in a mechanism independent of capsule production.

Capsular polysaccharides (CPS) are synthesized, transported and anchored to the surface of the cell by many bacterial species, forming a hydrated layer around the cell that protects it from desiccation and environmental stress (1). The *Escherichia coli* K30 group I capsule is assembled via the Wyz-dependent biosynthesis system, and polymerized and transported via the action of the Wza, Wzb and Wzc proteins (3). Wza is found in the outer membrane and polymerizes to form a channel through which the CPS is translocated (2). Wzc is an integral membrane protein of the inner membrane, and participates in the polymerization of CPS through its tyrosine autokinase activity. Wzb is found in the cytoplasm and is the cognate phosphatase of Wzc. (2). Whitfield *et. al.* developed an *E. coli* K30 group I mutant strain, CWG655Δ[*wza-wzb-wzc*$_{K30}$], that has a chromosomal deletion of the *wza*, *wzb* and *wzc* genes resulting in a mutant that exhibits decreased surface assembly of group I CPS when compared to the isogenic parental strain E69 (3).

Previous work suggests that the barrier function of the capsule may confer a level of antibiotic resistance by inhibiting access of the antibiotics to the cell (4,5). These studies have demonstrated that exposure of *E. coli* strains to sub-inhibitory concentrations of antibiotics results in an increase in CPS production and a corresponding increase in antibiotic resistance (4). However, there has been conflicting evidence surrounding the direct role of capsule in mediating antibiotic resistance. For example, Ganal *et. al.* reported resistance to kanamycin and streptomycin in a capsule-dependent fashion (6). In addition, Al Zharani *et. al.* found that the *E. coli* capsule was necessary for kanamycin resistance (7) and Song *et. al*. reported that capsule could interact with tetracycline, providing resistance via an unknown mechanism (8). Conversely, Parmar *et. al* found that the capsule did not confer resistance to kanamycin or tetracycline, while Drayson *et. al* concluded that antibiotic resistance following exposure to sub-inhibitory antibiotic concentrations was conferred in a capsule-independent fashion (9, 10). It has been suggested by several groups that capsule involvement in antibiotic resistance is antibiotic class specific, which may explain, in part, the varied and contradictory results seen in previous work (6-10).

Each of the many classes of antibiotics has a unique size, structure and bacterial target (11). The macrolide family is characterized by the presence of a large 14, 15, or 16-membered lactone ring and attached sugar groups (12). Different macrolides vary in ring size and in the chemical groups attached to the ring or sugar moieties (12). Macrolides of interest in this study include erythromycin, a common representative macrolide, as well as its derivatives clarithromycin and roxithromycin. Additionally, a new sub-group of macrolides called ketolides has been recently developed that includes the antibiotic telithromycin (12).

Macrolides act by binding to the 50s subunit of the bacterial ribosome at the 23s rRNA and inhibit protein synthesis by inducing dissociation of peptidyl-tRNA (13). Four main mechanisms of macrolide resistance have been previously observed. Firstly, the outer membrane of many Gram-negative bacteria can confer resistance (14). For example, mutations that impair the barrier function of the outer membrane were found to increase susceptibility to

azithromycin, clarithromycin and roxithromycin (15). Secondly, modification of the antibiotic target through methylation of the 23s rRNA can confer resistance (14). Thirdly, resistance can be conferred through an efflux pump (14). Lastly, macrolides can be inactivated by enzymatic activity in the cell, including that of esterases and phosphotransferases (14).

Given the conflicting evidence surrounding the role of capsule polysaccharides in antibiotic resistance, we examined the role of capsule production on antibiotic resistance to a range of antibiotics. We examined the *E. coli* K30 group I mutant strain CWG655Δ[*wza-wzb-wzc*$_{K30}$] in addition to its wild type (WT) parental strain E69 (3). We quantified the capsule production of both strains and compared the susceptibility of the two strains to ten different classes of antibiotics. From this, we identified macrolides as a class of interest and further examined the susceptibility of the strains to additional macrolides and a ketolide. By examining different macrolide antibiotics as well as a ketolide, we were able to determine if patterns of antibiotic susceptibility or resistance were specific to an individual antibiotic or if they applied to the larger antibiotic class.

We observed that CWG655Δ[*wza-wzb-wzc*$_{K30}$] produced diminished capsule compared to the WT and showed increased resistance to macrolide antibiotics. Overall, our results suggest that, for macrolide antibiotics, the *E. coli* K30 group I capsule does not play a role in antibiotic resistance, and that CWG655Δ[*wza-wzb-wzc*$_{K30}$] is resistant to macrolides via a mechanism independent of capsule but related to the absence of the *wza*, *wzb* and *wzc* genes.

MATERIALS AND METHODS

Bacterial Strains, Preparation of Media and Growth Conditions. *E. coli* K30 strains E69 (serotype: O9a:K30:H12) and CWG655 [*wza*$_{22\ min}$::*aadA* Δ(*wza-wzb-wzc*)$_{K30}$::*aphA3* Km^r Sp^r] were obtained from the laboratory of Dr. Chris Whitfield (Department of Molecular and Cellular Biology, University of Guelph). CWG655Δ[*wza-wzb-wzc*$_{K30}$] has a polar *aadA* insertion in the *wza* locus corresponding to 22 minutes on the *E. coli* K12 lineage map that eliminates expression of this copy of the *wza-wzb-wzc* locus (3). The second locus of *wza-wzb-wzc* was inactivated using PCR amplification and cloning into the suicide vector pWQ173, which was used to excise parts of *wza* and *wzc* as well as all of *wzb* (3). In this paper, strain CWG655 is referred to as either CWG655 Δ[*wza-wzb-wzc*]$_{K30}$ or as "mutant strain" while E69 is denoted as "wild type" (WT). All experiments were performed at either 21°C or 37°C. Liquid cultures were incubated on a shaker contained in either a 37°C walk-in incubator or at room temperature (approximately 21°C). Plates were incubated in either a 37°C walk-in incubator or at room temperature (approximately 21°C). Bacterial cells were grown in either Luria Bertani (LB) broth (1.0% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl, pH 7) or Mueller 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 as well as capsule staining. For other capsule staining experiments, as well as for the disc-diffusion assay, bacterial cells were grown on plates made from either LB (1.5% agar) or MH (1.7% agar) media.

Capsule Extraction and Quantification. Capsule extraction and quantification was performed as outlined by Brimacombe *et al.* (16), with slight modifications. A colony of each cell type was inoculated in 5 ml of either LB or MH media and grown overnight at either 21ºC or 37ºC. We conducted experiments using both LB and MH media because past work by Parmar *et. al.* identified differences in the production of capsular polysaccharide between strains grown in LB and MH media (9). The following day, optical density readings at 660nm for each culture were measured using a Spectronic 20+ spectrophotometer, and 1 ml of the same culture was transferred into a sterile microcentrifuge tube. Next, the 1ml 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 washed 3 times with 1ml of 50 mM NaCl. Next, the pellets were re-suspended in 1ml of 50 mM EDTA. The samples were then incubated at 37ºC on a shaker for 30min. After incubation, the samples were pelleted at 16,100 x g and the supernatant containing capsular polysaccharides was transferred into a sterile microcentrifuge tube. The subsequent capsule quantification was performed with the phenol-sulphuric acid assay (16). 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 curve. For capsule quantification, 400 uL of supernatant was combined with 400 uL of 5% phenol and 2 mL of 93% sulphuric acid in a glass test tube. Colour was allowed to develop for 10 min and the absorbance was measured at 490nm on a Spectronic 20+ spectrophotometer. Each experiment was done in replicates of three.

Capsule Staining. A colony of each cell type was streaked onto either LB or MH solid media and grown overnight at either 21ºC or 37ºC. Colonies were taken from the plates using a sterilized loop and suspended in 250uL of sterile saline. Capsule staining was performed using a modified version of the Maneval's capsule staining method described by Hughes and Smith (17). First, the cell suspension in sterile saline was mixed with 250µL of Congo Red (1% aqueous solution, Sigma Chemical Company C-6767), spread onto a glass microscope slide using a sterilized loop, and air-dried for 5-10 minutes. Next, 150µL of Maneval's solution was then pipetted onto the dried smears (0.047% w/v acid fuchsin, JT Baker Chemicals, A355-3; 2.8% w/v ferric chloride, Fisher Scientific I-89; 4.8% v/v aqueous glacial acetic acid, Acros, 42322-0025; 3.6% v/v aqueous phenol solution, Invitrogen IS509-037) and allowed to sit for approximately 2 minutes. The counterstain was washed off with dH_2O and the slides were air-dried before being viewed using a light microscope at 1000x magnification with oil immersion

Disc Diffusion Assay. Disc diffusion assays were performed using a modified version of the Kirby-Bauer method (18). Strains were grown overnight in liquid culture of LB or MH media at 21°C or 37°C. The optical density of the cultures was measured at 660nm using a Spectronic 20+ spectrophotometer and the cultures were then diluted with sterile broth to ~1 optical density unit. LB or MH plates were spread plated with 100µL of the diluted liquid cultures. Antibiotic discs (7mm diameter) prepared with either amoxicillin, nitrofurantoin, cefazolin, sulfamethoxazole, polymyxin, vancomycin, erythromycin, tetracycline, gentamycin, or norfloxacin (AB-biodisk) obtained from the Department of Microbiology and Immunology at UBC were placed onto the plates using sterilized forceps. For the roxithromycin, clarithromycin, and telithromycin disc diffusions, stock solutions of 10mg/mL roxithromycin, clarithromycin and telithromycin were obtained from the lab of Dr. Charles Thompson (Department of Microbiology and Immunology, UBC) and 10µL of each solution was pipetted onto blank discs. Each experiment was done is replicates of three, with three or four discs per plate. The plates were incubated for 18 hours at either 21°C or 37°C depending on the initial incubation temperature of the liquid culture, and the diameters of the zones of inhibition were

measured in millimetres. An increase in the diameter of the zone of inhibition indicates increased susceptibility and a decrease in the size of the zone of inhibition indicates increased resistance.

Statistical Analysis. Statistical analysis was performed for the disc diffusion assay as well as for the phenol-sulphuric acid assay. Statistical significance was determined using an unpaired, two tailed t-test ($p \leq 0.5$). For the phenol-sulphuric acid assay, comparisons were made between CWG655Δ[*wza-wzb-wzc*$_{K30}$] and the WT strain, at both 21ºC and 37ºC for LB and MH media. Comparisons were also made between 21°C and 37°C for the WT and CWG655Δ[*wza-wzb-wzc*$_{K30}$]. For the disc diffusion assay, comparisons were made between CWG655Δ[*wza-wzb-wzc*$_{K30}$] and the WT strain, at both 21ºC and 37ºC for LB and MH media.

RESULTS

Deletion of the *wza-wzb-wzc* genes decreases capsule production at 21°C but not at 37°C compared to the WT strain. To confirm decreased capsule biosynthesis ability of CWG655Δ[*wza-wzb-wzc*$_{K30}$] compared to the WT strain, we quantified capsular polysaccharide production of both strains at 21°C and 37°C using the phenol-sulphuric acid assay. Given that past groups have observed decreased capsule production at 37°C, compared to 21°C, we decided to conduct our analysis at both temperatures (9). We expected that CWG655Δ[*wza-wzb-wzc*$_{K30}$] would produce less capsular polysaccharide compared to the WT and that both strains would produce more capsular polysaccharide at 21°C, compared to 37°C. At 21°C, the WT strain exhibited 14-times greater production of capsular polysaccharide compared to CWG655Δ[*wza-wzb-wzc*$_{K30}$] when grown in LB broth (Fig. 1). At 37°C, we found no significant difference in capsular polysaccharide production for the WT compared to CWG655Δ[*wza-wzb-wzc*$_{K30}$] (Fig. 1). Additionally, we observed 12-times greater production of capsular polysaccharide for the WT strain at 21°C compared to 37°C (Fig. 1). We did not observe a significant difference in capsular polysaccharide production for CWG655Δ[*wza-wzb-wzc*$_{K30}$] between 21°C and 37°C (Fig.1). We replicated these experiments using both strains grown in MH broth and observed a similar trend in which the WT produced more capsular polysaccharide than CWG655Δ[*wza-wzb-wzc*$_{K30}$] (Supplemental Fig. 1). When grown in MH media we observed a less pronounced difference in polysaccharide production between the two strains at 21°C, indicating that LB would be a more suitable media for further study regarding the effects of the *wza-wzb-wzc* gene deletion on antibiotic resistance. From these results, we conclude that the WT strain produces more capsular polysaccharide than CWG655Δ[*wza-wzb-wzc*$_{K30}$] at 21°C, but not 37°C.

WT cells exhibit increased capsule thickness compared to CWG655Δ[*wza-wzb-wzc*$_{K30}$] on solid LB agar. To further confirm that CWG655Δ[*wza-wzb-wzc*$_{K30}$] was deficient in capsule compared to the WT, we performed capsule staining using Maneval's staining procedure, and visualized capsule size using light microscopy. Given that the antibiotic disc diffusion experiments were to be performed on solid media but the phenol-sulphuric acid assay used cells grown in liquid

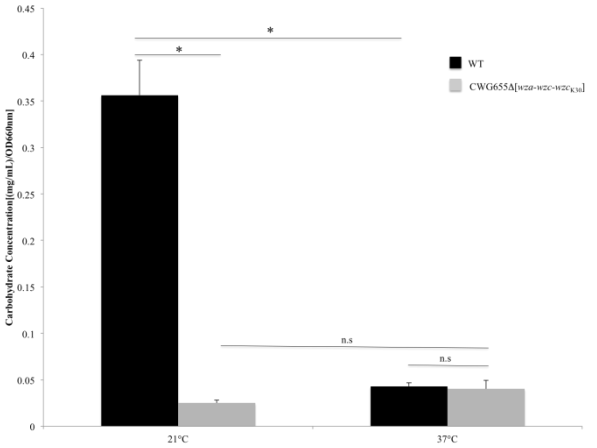

FIG 1 Differences in capsular polysaccharide produced by the WT strain and CWG655Δ[*wza-wzb-wzc*$_{K30}$] using the phenol-sulphuric acid capsule quantification method. Strains were cultured overnight in 21°C or 37°C shaking incubators in LB liquid media, and capsule polysaccharide was extracted and quantified using the phenol-sulphuric acid assay. * indicates $p<0.05$, n.s. indicates not significant.

culture, this experiment confirmed that differences in capsule production between the strains were also observed on solid media. Based on the phenol-sulphuric acid assay results (Fig. 1), we expected that the WT cells would have a larger visible capsule than the CWG655Δ[*wza-wzb-wzc*$_{K30}$] cells at 21°C, but not 37°C. Resulting images of stained WT cells (Fig. 2A) and CWG655Δ[*wza-wzb-wzc*$_{K30}$] cells (Fig. 2B) grown at 21°C showed increased capsule size visible around the WT cells, and not the CWG655Δ[*wza-wzb-wzc*$_{K30}$] cells. Both WT (Fig. 2C) cells and CWG655Δ[*wza-wzb-wzc*$_{K30}$] cells showed comparable capsule size at 37°C (Fig. 2D). However, we observed only minor differences in capsule size between WT cells grown at 21°C and 37°C (Fig. 2A, 2C). These results are unexpected given that we observed that the WT produced more capsular polysaccharides at 21°C, compared to 37°C (Fig. 1). We suspect that our inability to detect large differences in capsule size is due to disparate microscope image quality. Despite our inability to detect large difference in capsule size between WT cells grown at 21°C and 37°C, from these results we conclude that, when grown on solid media, CWG655Δ[*wza-wzb-wzc*$_{K30}$] shows decreased capsule size compared to the WT at 21°C, and that CWG655Δ[*wza-wzb-wzc*$_{K30}$] and the WT show similar capsule sizes at 37°C.

CWG655Δ[*wza-wzb-wzc*$_{K30}$] exhibits increased resistance to erythromycin compared to the WT strain. Due to the increase in capsule production observed for the WT strain compared to CWG655Δ[*wza-wzb-wzc*$_{K30}$] at 21°C, we hypothesized that an increase in capsular polysaccharides might influence antibiotic resistance in a class-dependent manner. In addition, differences in antibiotic susceptibility between the WT strain and CWG655Δ[*wza-wzb-wzc*$_{K30}$] were predicted to be more prominent at 21°C when compared to 37°C, due to the lack of differential capsule production between the strains

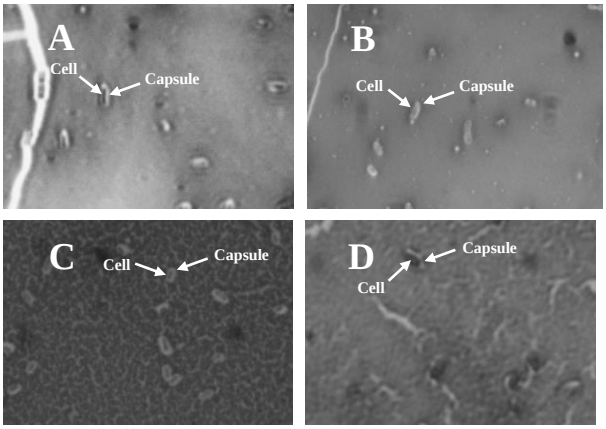

FIG 2 Differences in capsule thickness of WT and CWG655Δ[*wza-wzb-wzc*$_{K30}$] cells grown on solid LB agar media at 21°C and 37°C. (A) E69 WT cells grown on LB agar at 21°C; (B) CWG655Δ[*wza-wzb-wzc*$_{K30}$] cells grown on LB agar at 21°C. (C) E69 WT cells grown on LB agar at 37°C. (D) CWG655Δ[*wza-wzb-wzc*$_{K30}$] cells grown on LB agar at 37°C. Strains were grown overnight on LB agar plates at 21°C, and cell capsules were stained using Maneval's staining protocol and visualized at 1000x magnification. Grey regions indicate cell bodies, and white regions indicate capsule.

observed at 37°C (Fig. 1). To test our hypothesis we conducted a screen of ten antibiotics, each of a different antibiotic class, using an antibiotic disc diffusion assay on LB and MH agar media for both strains at 21°C and 37°C. We observed that CWG655Δ[*wza-wzb-wzc*$_{K30}$] exhibited decreased resistance to some antibiotics, such as nitrofurantoin, yet no consistent trends in resistance changes to many other antibiotics, when compared to the WT (Supplemental Fig. 2). However, we also observed that CWG655Δ[*wza-wzb-wzc*$_{K30}$] exhibited increased resistance to some antibiotics when compared to the WT, such as erythromycin and tetracycline (Supplemental Fig. 2). These results suggest that a deletion of the *wza-wzb-wzc* genes can increase, decrease, or have no effect on resistance to antibiotics, depending on the antibiotic tested.

Based on the observed results, we identified erythromycin as an antibiotic of interest for further study. At 37°C, the WT strain had some degree of susceptibility to erythromycin, as indicated by the presence of a zone of inhibition around the erythromycin disc, while CWG655Δ[*wza-wzb-wzc*$_{K30}$] showed no susceptibility, growing consistently up to the edge of the disc (Fig. 3A). The zones of inhibition surrounding the erythromycin discs appeared as a gradient, not a distinct line (Fig. 3A).

CWG655Δ[*wza-wzb-wzc*$_{K30}$] showed a significant increase in resistance to the erythromycin compared to the WT strain (Fig. 3B). We observed a similar pattern at 21°C, but at this temperature results did not reach significance (Fig. 3B). Additionally, we obtained similar results with a disc diffusion assay performed on MH agar, where CWG655Δ[*wza-wzb-wzc*$_{K30}$] exhibited significantly increased erythromycin resistance at 37°C and a similar but less pronounced result at 21°C compared to the WT strain (Supplemental Fig. 3).

We observed no significant differences in capsule production at 37°C between CWG655Δ[*wza-wzb-wzc*$_{K30}$] and the WT strain (Fig. 1). However, we observed significant differences in resistance between CWG655Δ[*wza-wzb-wzc*$_{K30}$] and the WT strain at 37°C (Fig. 3). These results suggest that the differential susceptibility of the strains to erythromycin may not be due to the physical presence of capsule. From this we conclude that although the deletion of the *wza-wzb-wzc* capsule biosynthesis genes confers increased resistance to erythromycin, this effect may not be due to decreased capsule production.

Resistance of CWG655Δ[*wza-wzb-wzc*$_{K30}$] to erythromycin extends to the macrolides clarithromycin and roxithromycin, but not to the ketolide telithromycin. We observed differences in antibiotic susceptibility between the WT strain and CWG655Δ[*wza-wzb-wzc*$_{K30}$] that varied with antibiotic class (Supplemental Fig. 2). Additionally, we observed that CWG655Δ[*wza-wzb-wzc*$_{K30}$] exhibited increased resistance to erythromycin compared to the WT (Fig. 3). In order to determine if the resistance conferred by the *wza-wzb-wzc* gene deletion was specific to erythromycin or if it also applied to other antibiotics in the macrolide class, we conducted further disc diffusion assays using clarithromycin and roxithromycin. Additionally, we used telithromycin, which is a member of the macrolide sub-group the ketolides. We examined the susceptibilities of the WT strain and CWG655Δ[*wza-wzb-wzc*$_{K30}$] at both 21°C and 37°C. At 21°C, disc diffusion results showed a 3-fold increase in susceptibility to roxithromycin for the WT strain compared to CWG655Δ[*wza-wzb-wzc*$_{K30}$]. A similar trend of increased susceptibility of the WT strain was seen for clarithromycin, but these results were not significant at 21°C. (Fig 4). When grown at 37°C, we observed at 10-fold increase in susceptibility to roxithromycin for the WT compared to CWG655Δ[*wza-wzb-wzc*$_{K30}$] (Fig. 4). Similarly, we observed that the WT was susceptible to clarithromycin with a clear zone of inhibition around the antibiotic disc, while CWG655Δ[*wza-wzb-wzc*$_{K30}$] was resistant with growth up to the edge of the antibiotic disc. At both temperatures, we observed that the WT and CWG655Δ[*wza-wzb-wzc*$_{K30}$] were comparably resistant to the ketolide, telithromycin (Fig. 4). We observed a similar trend in results when disc diffusion assays were replicated on MH media (Supplemental Fig. 4). From these results we conclude that the *wza-wzb-wzc* gene deletion confers increased resistance to the macrolides clarithromycin and roxithromycin, similar to the pattern seen in erythromycin, but this does not extend to the ketolide telithromycin. This effect is independent of physical capsule presence.

DISCUSSION

In this study, comparison of capsule production between the WT strain and CWG655Δ[*wza-wzb-wzc*$_{K30}$] revealed that CWG655Δ[*wza-wzb-wzc*$_{K30}$] exhibited 14-times less CPS production than the WT strain at 21°C . This is consistent with the expected

results, due to the chromosomal deletion of the capsule biosynthesis genes *wza*, *wzb* and *wzc*, as described by Whitfield *et al.* (3). Our results are consistent with the findings of Parmar *et al.*, who examined a strain deficient in only the Wza channel-forming protein necessary for capsule assembly, and demonstrated decreased CPS production by that mutant strain (9).

We observed a greater difference in capsule production between the WT strain and CWG655Δ[*wza-wzb-wzc*$_{K30}$] at 21°C than at 37°C, and an overall increase in capsule production at 21°C for the WT (Fig. 1). Although the group I *E. coli* capsule was not previously thought to be thermoregulated (2), studies by Parmar *et. al.*, Drayson *et. al*, and Stout *et. al*., and have reported increased capsule production at 21°C compared to 37°C (9, 10, 19). This observation is of interest because some previous studies that have failed to find differences in antibiotic resistance based on the presence or absence of capsule carried out experiments at only 37°C (20). For example, Naimi *et. al.* examined the role of capsule in streptomycin susceptibility with organisms grown at 37°C and observed no difference in susceptibility between a *wza* mutant and its isogenic WT strain (20). A possible explanation for their result is that the strains were producing comparable levels of polysaccharides at 37°C. However, other studies have examined the same strains at both 21°C and 37°C and also failed to find differences in antibiotic resistance to streptomycin between WT and capsule deficient mutants (9, 10). The previous findings on the topic of capsule-dependent antibiotic resistance are contradictory, however, as there have been other groups that have suggested a link between capsule production and resistance to certain antibiotics, such as kanamycin, tetracycline, and streptomycin (4, 7, 8). Therefore, we suggest that capsule may influence antibiotic resistance for specific classes of antibiotics.

In this study, we used a screen of ten different antibiotics to compare the susceptibilities of the WT strain and CWG655Δ[*wza-wzb-wzc*$_{K30}$] using the disc diffusion assay for antibiotic resistance. Due to the limited number of replicates we performed, the majority of antibiotics tested showed no significant difference in susceptibility between the two strains (Supplementary Fig. 2). Indeed, at 21°C we noticed no significant differences between the antibiotic susceptibilities of either strain for any of the antibiotics tested. Nonetheless, the general trend supports the observation in the literature that the presence of an intact capsule can increase resistance to a variety of antibiotics (4, 7, 8), or have no effect (9, 10). However, we also identified another possibility: the absence of *wza*, *wzb*, and *wzc* in our mutant may increase antibiotic resistance. We demonstrated that at 37°C CWG655Δ[*wza-wzb-wzc*$_{K30}$] exhibited an increase in resistance to both tetracycline and erythromycin

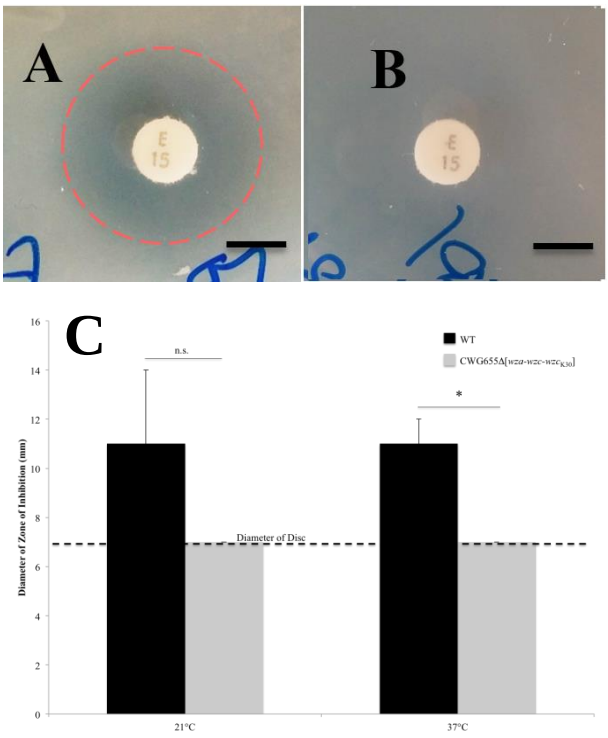

FIG 3 Susceptibility of CWG655Δ[*wza-wzb-wzc*$_{K30}$] and the WT strain to erythromycin via disc diffusion assay. (A, B) Representative disc diffusion results showing that the WT is susceptible to erythromycin, as seen by a zone of clearance around the erythromycin disc indicated by a red dashed circle. CWG655Δ[*wza-wzb-wzc*$_{K30}$] is shown to be resistant to erythromycin, as seen by the lack of inhibition around the erythromycin disc. Scale bars = 7mm; (C) Differences in susceptibility of the WT and CWG655Δ[*wza-wzb-wzc*$_{K30}$] to erythromycin at 21°C and 37°C. Disc diffusion assays were carried out using antibiotic discs on LB agar plates. An increase in the diameter of the zone of inhibition indicates an increase in susceptibility. * indicates $p<0.05$, n.s. indicates non-significant. Dashed line indicates diameter of antibiotic disc.

compared to the WT strain (Supplementary Fig. 2). Our results indicate that our understanding of the role of capsule in antibiotic resistance should be modified to suggest that an increase in capsule production can either increase, decrease or have no effect on antibiotic resistance depending upon the antibiotic class being examined.

Due to the increase in resistance observed by CWG655Δ[*wza-wzb-wzc*$_{K30}$] to erythromycin, we decided to further investigate macrolides as our antibiotic family of focus. In this study, we demonstrated that CWG655Δ[*wza-wzb-wzc*$_{K30}$] showed increased resistance to the macrolide antibiotics erythromycin, clarithromycin, and roxithromycin, but not the ketolide antibiotic telithromycin, when compared to the WT strain (Fig. 3, 4). We observed comparable results at 21°C and 37°C. However, differences in susceptibility to erythromycin and clairithromycin between the WT and CWG655Δ[*wza-wzb-wzc*$_{K30}$] only reached significance at 37°C (Fig. 3, 4). We also observed that there were no significant

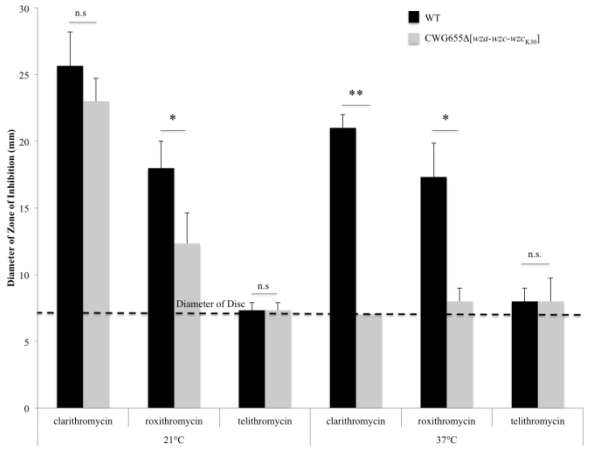

FIG 4 Differences in susceptibility of the WT strain and CWG655Δ[*wza-wzb-wzc*$_{K30}$] to the macrolides clarithromycin, roxithromycin, and the ketolide telithromycin at 21°C and 37°C. Disc diffusion assays were carried out on LB agar plates. An increase in the diameter of the zone of inhibition indicates an increase in susceptibility. * indicates $p<0.05$, ** indicates $p<0.005$, n.s. indicates non-significant. Dashed line indicates diameter of antibiotic disc.

differences in CPS production at 37°C between the WT and CWG655Δ[*wza-wzb-wzc*$_{K30}$] (Fig. 1). Taken together these results suggest that the presence or absence of capsule does not play a role in antibiotic resistance to macrolides for these strains, but that the absence of the *wza*, *wzb* and *wzc* genes may play a role in the increase in resistance of CWG655Δ[*wza-wzb-wzc*$_{K30}$]. These results are consistent with past observations made by Drayson *et. al* which suggested that antibiotic resistance can be conferred in a capsule-independent fashion (10). Given that our data suggest that the absence of *wza*, *wzb* and *wzc* increases antibiotic resistance in a capsule-independent manner, a discussion of the potential mechanisms of resistance will follow.

A variety of mechanisms for resistance to macrolides have been observed (21). Given that capsule is associated with the outer membrane (OM) and *wza*, *wzb* and *wzc*, are involved in capsule assembly we suggest that the mechanism of most relevance to this study is the role of the OM as a permeability barrier. Typically, macrolide antibiotics are used to treat Gram-positive infections because the OM of Gram-negative bacteria can confer a level of resistance that makes clinical use of macrolides, particularly erythromycin, challenging for those types of infections (22). This is thought to be due to the hydrophobic nature of the macrolides, which can prevent them from passing the charged lipid A component of LPS present in the OM (22).

Because our mutant strain lacks three genes and their corresponding protein products, we cannot identify a single gene product that, when absent, confers the observed increase in macrolide resistance of CWG655Δ[*wza-wzb-wzc*$_{K30}$]. However, we have developed a model as a potential explanation for the increased resistance observed in CWG655Δ[*wza-wzb-wzc*$_{K30}$] in the context of OM permeability. Whitfield *et. al.* observed that the CWG655Δ[*wza-wzb-wzc*$_{K30}$] strain shows depleted surface assembly of CPS (3). These results were replicated in this study (Fig.1, 2). Whitfield *et. al.* also observed that CWG655Δ[*wza-wzb-wzc*$_{K30}$] produces capsular oligosaccharides with a low degree of polymerization that are attached to the lipid A moiety of LPS and form an alternate glycoform of LPS called K-LPS (3). We speculate that the increased resistance to macrolides exhibited by CWG655Δ[*wza-wzb-wzc*$_{K30}$] to macrolides may be related to the formation of K-LPS, and therefore an altered OM structure and permeability (C. Whitfield, personal correspondence).

This notion is supported by previous studies that have found that modifications in OM permeability alter macrolide susceptibility. Vaara found that mutations that affected OM structure and increased permeability, such as mutations in lipid A synthesis in *E. coli*, decreased the MICs of erythromycin, roxithromycin, clarithromycin and azithromycin (23). Similarly, Buyuk *et. al.* found that certain strains of *Pseudomonas aeruginosa* are more susceptible to macrolides due to increased membrane permeability (45). Finally, Farmer *et. al* observed that the MIC of azithromycin was increased 8 times with the addition of a magnesium supplementation that decreased membrane permeability (25). These previous findings lend support to our proposed model wherein the *wza-wzb-wzc* deletion confers macrolide resistance through an alteration of the OM structure that causes changes in OM permeability.

The last observation of significance in this study is that both the WT strain and CWG655Δ[*wza-wzb-wzc*$_{K30}$] exhibited comparable levels of resistance to telithromycin, with but differing levels of susceptibility to macrolides that were close derivatives of erythromycin (Fig. 3, 4). Different macrolide antibiotics vary in chemical components that are attached to the lactone ring or sugar moieties (11). Clarithromycin is derived from erythromycin by substituting a methoxy group for the C-6 hydroxyl group of erythromycin (26), while roxithromycin has an N-oxime side chain attached to the lactone ring (27). Telithromycin is a member of a macrolide derivative family called ketolides, which have a further modified structure from typical macrolides in that a keto functional group is substituted for the sugar moiety at C-3 on the lactone ring (26). A methoxy group replaces the hydroxyl group at C-6 and C-11-12 is cyclized to make a carbamate group with an imidazo-pyridyl group attachment (26). We suggest that the difference we see in susceptibility may be due to structural differences between telithromycin and the macrolides. Most literature in this area suggests that ketolides have increased activity against Gram-negative pathogens compared to macrolides; however, limited data is available regarding

mechanisms of resistance to ketolides (26). Recent work has reported that few bacterial strains exhibit telithromycin resistance (26). In the literature, it appears that those strains that are resistant to ketolides exhibit their resistance through mechanisms that are common to those for macrolide resistance. Walsh *et al.* reported two mechanisms of telithromycin resistance: target modification by methylation of the 23s rRNA by the *erm*(B) methylase gene or efflux mediated by the *mef*(B) gene (28). With our limited data we cannot develop any conclusive hypotheses regarding the source of telithromycin resistance in the WT and CWG655Δ[*wza-wzb-wzc*$_{K30}$]. However, possible mechanisms include exclusion due to its size or charge, or the presence of any of the resistance mediating genes described above.

In this study our aim was to examine the CWG655Δ[*wza-wzb-wzc*$_{K30}$] mutant strain and its WT parental strain E69, with particular focus on the role of the K30 group I capsule in antibiotic resistance to multiple antibiotic families. We observed that CWG655Δ[*wza-wzb-wzc*$_{K30}$] produced less capsular polysaccharide than the WT at 21°C, but not 37°C. We also observed that CWG655Δ[*wza-wzb-wzc*$_{K30}$] showed increased resistance to macrolides, but not the ketolide subgroup, when compared to the WT at 21°C and 37°C. Taken together, our data suggest that a deletion of the *wza*, *wzb* and *wzc* genes confers resistance to macrolide, but not ketolide, antibiotics via a capsule-independent mechanism. Overall, we conclude that the absence of the capsule biosynthesis genes *wza*, *wzb* and *wzc* confers increased resistance to macrolide antibiotics.

FUTURE DIRECTIONS

Although we have indicated that the absence the *wza*, *wzb*, and *wzc* genes is important for macrolide resistance, and that this phenomenon is capsule-independent, the mechanism of resistance remains unknown. The clearest and most pressing direction to be taken from this study is an examination of the effect of single gene mutations in *wza*, *wzb* or *wzc* on resistance to macrolide family antibiotics. Since this study was conducted using a mutant with a deletion of all three of these capsule assembly genes, a causal relationship cannot be established between the absence of any one gene (or a combination) and the observed increase in resistance to macrolides. Further study is warranted to determine whether or not the observed effect can be traced to a single gene product, or whether the effect is the result of a combination of deletions. Additionally, the effect of other outer membrane genes could be studied in order to determine whether this phenomenon is unique to the capsule biosynthesis genes *wza*, *wzb* and *wzc* or whether deletions in other membrane channels, kinases, etc. are sufficient to induce the observed, macrolide-resistant phenotype.

We observed that the WT strain and CWG655Δ[*wza-wzb-wzc*$_{K30}$] exhibited telithromycin resistance. Future work could also focus on elucidating the mechanism of resistance to telithromycin used by both E69 and CWG655Δ[*wza-wzb-wzc*$_{K30}$]. Given that the incidence of telithromycin is rare, future studies could attempt to explain the observed resistance. For example, the strains could be examined in an attempt to determine if they harbour genes that have been identified in the literature as being involved in telithromycin resistance (28).

We observed that the WT strain produced 12-times greater levels of capsular polysaccharides at 21°C compared to 37°C. Past publications have reported similar results (9). Given that group I capsule production is not known to be thermoregulated (2), future work could examine the potential mechanisms behind these results by examining the expression of genes involved in capsule assembly at 21°C and 37°C.

Finally, future experiments could be conducted to further examine the other observed results in our antibiotic screen. That is, higher replicate screens could, and should, be conducted in order to determine the significance of observed trends, and to further confirm the results observed herein. Moreover, certain antibiotics could be further studied in order to aid in the explanation of the results. For example, tetracycline resistance was increased in the absence of capsule in this study and further work could focus on elucidating this mechanism. Additionally, for norfloxacin at 21°C the WT strain showed increased resistance compared to CWG655Δ[*wza-wzb-wzc*$_{K30}$], but this result was reversed at 37°C. Future work could focus on attempting to replicate this result and examine the mechanism behind it. Indeed, these are but two of several examples of antibiotics that could be further studied in relation to the role of capsule in antibiotic resistance.

ACKNOWLEDGEMENTS

We would like to thank the Department of Microbiology and Immunology at the University of British Columbia for their funding and support. We would like the thank Dr. David Oliver, Jia Wang and Céline Michaels, for their guidance, instruction, and support, as well as the staff of the media room for providing us with equipment and supplies. In addition, we would like to thank the Department of Pharmacology at UBC for gifting the Department of Microbiology with many of the antibiotic disks used in our experiments. Further, we would like to thank Dr. Chris Whitfield at the University of Guelph for providing the *E. coli* strains and for his valuable insight into our work. Finally we would like Dr. Charles Thompson at the University of British Columbia for providing us with the macrolide antibiotics used in our experiments.

REFERENCES

1. **Reid, AN, Szymanski, CM.** 2010. Biosynthesis and assembly of capsular polysaccharides-Chapter 20, p. 351-373. *In* Moran, A (ed), Microbial Glycobiology: Structure, Relevance and Application. Elsevier Inc, London, UK.
2. **Whitfield, C.** 2006. Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli.* Annu. Rev. Biochem. **75:**39-68.
3. **Reid, AN, Whitfield, C.** 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.

4. **Lu, E, Trinh, T, Tsang, T, Yeung, J.** 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.
5. **Slack, MP, Nichols, WW.** 1982. Antibiotic penetration through bacterial capsules and exopolysaccharides. J. Antimicrob. Chemother. **10:**368-372.
6. **Ganal, S, Guadin, C, Roensch, K, Tran, M.** 2007. Effects of streptomycin and kanamycin on the production of capsular polysaccharides in *Escherichia coli* B23 cells. J. Exp. Microbiol. Immunol. **11:**54-99.
7. **Al Zahrani, F, Huang, M, Lam, B, Vafaei, R.** 2013. Capsule formation is necessary for kanamycin tolerance in *Escherichia coli* K-12. J. Exp. Microbiol. Immunol. **17:**24-28.
8. **Song, C, Sun, XF, Xing, SF, Xia, PF, Shi, YJ, Wang, SG.** 2013. Characterization of the interactions between tetracycline antibiotics and microbial extracellular polymeric substances with spectroscopic approaches. Environ. Sci. Pollut. Res. Int. **21**: 1786-1795.
9. **Parmar, S, Rajwani, A, Sekhon, S, Suri, K.** 2014. The *Escherichia coli* K12 capsule does not confer resistance to either tetracycline or streptomycin. J. Exp. Microbiol. Immunol.**18:**76-81.
10. **Drayson R, Leggat T, Wood M.** 2011. Increased antibiotic resistance post-exposure to sub-inhibitory concentrations is independent of capsular polysaccharide in *Escherichia coli*. J. Exp. Microbiol. Immunol. **15**:36-42.
11. **Walsh, C.** 2003. Antibiotics: actions, origins, resistance. American Society for Microbiology (ASM).
12. **Omura, S.** 2002. Macrolide antibiotics: chemistry, biology, and practice. Academic Press.
13. **Tenson, T, Lovmar, M, Ehrenberg, M.** 2003. The mechanism of action of macrolides, lincosamides and streptogramin B reveals the nascent peptide exit path in the ribosome. J. Mol. Biol. **330:**1005-1014.
14. **Leclercq, R.** 2002. Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. Clin. Infect. Dis. **34:**482-492.
15. **Vaara, M.** 1993. Outer membrane permeability barrier to azithromycin, clarithromycin, and roxithromycin in gram-negative enteric bacteria. Antimicrob. Agents Chemother. **37:**354-356.
16. **Brimacombe CA, Stevens A, Jun D, Mercer R, Lang AS, Beatty JT.** 2013. Quorum-sensing regulation of a capsular polysaccharide receptor for the *Rhodobacter capsulatus* gene transfer agent (RcGTA). Mol. Microbiol. **87**:802-817
17. **Hughes RB, Smith, A.** 2011. Capsule stain protocols. ML Microbe Library, ASM. Accessed on 9th March 2015 at http://www.microbelibrary.org/component/resource/laboratorytest/3041-capsule-stain-protocols.
18. **Hudzicki, J**. 2009. Kirby-Bauer disk diffusion susceptibility test protocol. ML Microbe Library, ASM. Accessed on 8th February 2015 at http://www.microbelibrary.org/component/resource/laboratory-test/3189-kirby-bauer-disk-diffusion-susceptibility-test-protocol.
19. **Stout, V, Gottesman, S**. 1990. RcsB and RcsC: a two-component regulator of capsule synthesis in *Escherichia coli*. J. Bacteriol. **172:**659-669.
20. **Naimi, I, Nazer, M, Ong, L, Thong, E.** 2009. The role of wza in extracellular capsular polysaccharide levels during exposure to sublethal doses of streptomycin. J. Exp. Microbiol. Immunol. Vol. **13:**36-40.
21. **Leclercq, R.** 2002. Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. Clin. Infect. Dis. **34:**482-492.
22. **Poole, K.** 2002. Outer membranes and efflux: the path to multidrug resistance in Gram-negative bacteria. Curr. Pharm. Biotechnol. **3:**77-98.
23. **Vaara, M.** 1993. Outer membrane permeability barrier to azithromycin, clarithromycin, and roxithromycin in gram-negative enteric bacteria. Antimicrob. Agents Chemother. **37:**354-356.
24. **Buyck, J, Tulkens, PM, Van Bambeke, F.** 2011. Increased susceptibility of *Pseudomonas aeruginosa* to macrolides in biologically-relevant media by modulation of outer membrane permeability and of efflux pump expression, p. 17-20. *In* Anonymous 51st Interscience conference on antimicrobial agents and chemotherapy, Chicago.
25. **Farmer, S, Li, ZS, Hancock, RE.** 1992. Influence of outer membrane mutations on susceptibility of *Escherichia coli* to the dibasic macrolide azithromycin. J. Antimicrob. Chemother. **29:**27-33.
26. **Zuckerman, JM.** 2004. Macrolides and ketolides: azithromycin, clarithromycin, telithromycin. Infect. Dis. Clin. North Am. **18:**621-649.
27. **Takashima, H.** 2003. Structural consideration of macrolide antibiotics in relation to the ribosomal interaction and drug design. Curr. Top. Med. Chem. **3:**991-999.
28. **Walsh, F, Willcock, J, Amyes, S.** 2003. High-level telithromycin resistance in laboratory-generated mutants of *Streptococcus pneumoniae.* J. Antimicrob. Chemother. **52:**345-353.