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Single Deletion of *Escherichia coli* K30 Group I Capsule Biosynthesis System Component, *wzb*, Is Not Sufficient to Confer Capsule-Independent Resistance to Erythromycin

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***Escherichia coli* overcomes different environmental stresses to survive, one of which is cellular death by antibiotics. The capsule is a high molecular weight bacterial surface polysaccharide layer, which is thought to confer resistance against antibiotics, perhaps by acting as a steric or charged barrier to entry. The Wzy system is responsible for the synthesis of group I capsule in *E. coli* K30. The *wzy* gene cassette is formed by three different genes: *wza*, *wzb* and *wzc*. Each protein expressed from the components of the *wzy* cassette serves its own specific function in bacterial capsule synthesis, which is capsule assembly and its secretion to the cell surface. It has been shown from previous studies that a complete knockout of the *wzy* gene cassette, as well as the knockout of the *wza* gene alone, is sufficient to confer a resistance phenotype against the macrolide antibiotic, erythromycin, in a capsule-independent manner. However, the mechanism behind this contradictory phenotype is not well known. We hypothesised that *wzb* may play a role in erythromycin resistance as it functions in the cytoplasm as a non-specific phosphatase, potentially inactivating macrolides by dephosphorylation. We examined resistance to erythromycin using five different strains, which were various mutants of the *wzy* cassette. Antibiotic resistance of the strains was tested using a disc diffusion assay and results were further supported by determining the minimum inhibitory concentrations. Our observations showed that the double knockout of *wzb* and *wza*, in addition to the single *wza* knockout, had the same resistance phenotype to erythromycin as the complete *wzy* knockout, while a single knockout of *wzb* did not. From these results, we conclude that the single deletion of the capsule biosynthesis gene *wzb* alone is not sufficient to confer the resistance phenotype to erythromycin.**

The capsule is a bacterial surface structure that is comprised of high molecular weight polysaccharides and individual chains of linear or branched polymers sometimes including non-carbohydrate moieties (1). It is known to be essential to many aspects of bacterial survival, which include phagocytosis by immune cells and desiccation (2). Previous studies have shown that the thick capsule layer may act as a diffusion barrier to antibiotics to confer a level of resistance (2). In *E. coli* K30, capsule formation is dependent on three genes of the *wzy* gene cassette, *wza*, *wzb*, and *wzc*, which produce the proteins to form the Wzy capsule polymerization system (3). The three parts of this system fulfill their respective roles by cooperatively polymerizing group I capsule and transporting it to its location on the cell surface (3). However, the mechanism by which the capsule assembles itself outside the cell is not well known (1). Once outside of the cell, the group I capsule provides a physical barrier to the environmental stresses and challenges the bacteria may encounter (2).

Wza forms a channel in the outer membrane and allows the translocation of polymerized polysaccharides to the cell exterior (3, 4). Wzc is an inner-membrane associated tyrosine autokinase that is involved in the polymerization of capsule (3, 4). Wzc works in conjunction with Wzb, which is a cognate Wzc phosphatase located in the cytoplasm (4). Although Wzc is a known substrate for Wzb, Wzb is also known to have non-specific tyrosine phosphatase activity (4).

Botros et al. have shown that deletion of all three genes in the *wzy* cassette of *E. coli* K30 resulted in an increased resistance of the strain against macrolide antibiotics in a capsule-independent manner (5). In a following study, Su et al. have shown that deletion of only the *wza* gene in the *wzy* cassette of *E. coli* K30 was sufficient to confer the phenotype of increased resistance against macrolide antibiotics (6). However, the specific role of Wzb in this phenotype of increased resistance against macrolide antibiotics has not been well elucidated. Macrolides are a class of large hydrophobic antibiotics that feature a large lactone ring (7). They deliver antibacterial activity by inhibiting bacterial protein synthesis (7). Nevertheless, bacteria have developed a spectrum of mechanisms to circumvent macrolide-derived inhibition. For example, an established method of macrolide inactivation that is often employed by bacteria is deactivation of the antibiotic through phosphorylation (8). Once a macrolide is phosphorylated by a cellular kinase, it is in its inactive form and remains as an inert molecule within the cell (8).

We hypothesised that Wzb, as a nonspecific phosphatase, would have a significant effect on capsule-independent macrolide resistance phenotype that was previously observed in *E. coli* K30 (5). We propose that the absence of *wzb*, a component of the *E. coli* K30 Wzy group I capsule polymerization system, will result in an increased resistance against macrolide antibiotics in a capsule-independent manner. Given that Wzb is a non-specific and cytosolic tyrosine phosphatase, phosphorylated macrolides could be a potential substrate (4). If this is the case, then deletion of *wzb* may result in lower levels of dephosphorylation of macrolides, which could result in higher levels of phosphorylated macrolides. Since phosphorylated macrolides are inactive, the cell will have evaded the protein

inhibition of macrolides and gained resistance. If the observed phenotype is consistent with our hypothesis, then we should see an increase in macrolide resistance with *wzb* deletion.

In order to investigate if the deletion of *wzb* is sufficient to convey the mentioned phenotype, we performed a series of assays with five *E. coli* K30 strains. To test this hypothesis, we used a disc diffusion assay using erythromycin, a type of macrolide, as well as a minimum inhibitory concentration assay, in order to determine the minimum inhibitory concentration of erythromycin for each strain. We used five different strains of *E. coli* K30 in our experiments: the wild-type (E69), the *wzy* complete knock-out (CWG655Δ), the *wza* and *wzb* double gene knock-out (CWG343), the *wza* single gene knock-out (CWG281) and the *wzb* single gene knock-out (CWG344) (9). Including variations of knock-out strains helped us to determine if *wzb* is sufficient for inducing capsule-independent macrolide resistance in bacteria and to compare the results to the two other components of the Wzy group I capsule polymerization system, *wza* and *wzc*.

MATERIALS AND METHODS

Bacterial strains, preparation of media, and growth conditions. *E. coli* K30 strains CWG343 (E69 with wzb_{cps}::*aph3A* and $wza_{22\ min}$ ::*aadA* insertion polar on wzb_{eps} and *etk*) and CWG344 (unpublished) were obtained from the laboratory of Dr. Chris Whitfield from the Department of Molecular and Cellular Biology, University of Guelph (9). *E. coli* wild type E69 (serotype O9a:K30:H12), CWG655Δ ($wza_{22\ min}$::*aadA* Δ(*wza-wzb-wzc*)$_{K30}$::*aphA3*) and CWG281 (which has a gentamycin resistance cassette inserted to inactivate *wza*) were obtained from the MICB 421 laboratory stock from the Department of Microbiology and Immunology (University of British Columbia). In all experiments, liquid cultures were incubated on a shaker in a 37˚C walk-in incubator. Cells were grown in Luria Bertani (LB) broth (1.0% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl, pH 7). For the disc diffusion assay, cells were grown on 1.5% agar LB plates and incubated at 37˚C.

Strain confirmation using PCR amplification of *wza*, *wzb* and *wzc*. The PCR amplification protocol was adapted from the protocol by Su et al. (6). The following primers flanking the *wzy* cassette were acquired from the Microbiology and Immunology Department, UBC at a concentration of 100 µM: 1d-Whit715-F 5'-CCTGGTCAGGGATCCAACAGTCTG-3' and 1d-Whit715-R 5'TCGCGGATCCAATTGTTACGA-3'. They were diluted to 10 µM using sterile distilled water before addition into the PCR reaction mix, as follows: 5 µl of 10X PCR Buffer (-$MgCl_2$), 1.5 µl of 50 mM $MgCl_2$, 1 µl of 10 mM dNTP mix, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 0.2 µl Taq DNA polymerase, a colony of the strains (E69, CWG281, CWG343, CWG344, or CWG655Δ), and 40.3 µl of sterile distilled water. A separate PCR reaction was prepared for each strain using the exact same reaction mix, but with an additional 1% DMSO in order to enhance PCR products as it reduces the secondary structures that interfere with the replication process (10). A positive control reaction with a colony from E69 was set up with the following primers flanking *ompc*: OmpC-2deltaF 5'-GCATTTACATTTTGAAACATCTATAGC-3' and OmpC-2deltaR 5'-GGGTTGTGGTTTTTGATCGC-3'. The following PCR protocol was used in the thermocycler: 94˚C for 120 seconds, 35 cycles of 30 seconds at 94˚C, 30 seconds at 50˚ C, and 6 minutes at 72˚C, and a final step of 72˚C for 10 minutes.

Gel electrophoresis for PCR product visualization. The PCR products were visualized on a 1% agarose gel, which was prepared by dissolving 1 g of agarose in 100 ml of 1X TAE and adding 10 µl of 10,000X SYBR safe DNA dye. DNA loading dye was added to each 50 µl PCR reaction to give a 1x final concentration, then a total of 30 µl of each sample was loaded in the appropriate wells. One µl of the 10X DNA loading dye was added to 9 µl of 1 Kb Plus DNA Ladder which was loaded into the first lane. The gel was run for 1 hour and 15 minutes at 115 V.

Disc diffusion assay. Disc diffusion assays were performed using a modified version of the Kirby-Bauer method as reported by Botros et al. (5). Bacterial strains were grown overnight in 10 ml of LB, shaking at 37˚C. A Spectronic 20+ spectrophotometer was then used to measure the optical density at 660 nm of each culture. All strains were diluted with sterile LB to ~0.8 OD_{660} before 100 µl of each strain was spread plated on an LB agar plate. Each plate was divided in half, with one half receiving three equally spread out erythromycin antibiotic discs (obtained from the Department of Microbiology and Immunology, UBC) and the other half receiving three blank Whatman paper discs of the same diameter (7 mm), which served as a negative control. The discs were placed on the LB agar plates using sterile forceps. The plates were incubated at 37˚C for 19 hours before the zone of inhibition was measured using a ruler (in mm). A visible zone of inhibition around the erythromycin discs indicates susceptibility to the antibiotic, while the absence of a zone inhibition indicates resistance to the antibiotic.

Determination of minimum inhibitory concentration (MIC) assay. We used the MIC assay to determine the minimum erythromycin concentration to inhibit the cellular growth. The MIC assay was performed using a modified protocol that was originally described by Su et al. (6). Bacterial strains were inoculated in 5 ml of LB broth and grown overnight at 37˚C for 24 hours. OD_{660} of overnight cultures was measured using Spectronic 20+ spectrophotometer and the cultures were diluted with LB broth to achieve OD_{660} of 0.0001. A stock solution of erythromycin (100 mg/ml) was made by adding 0.5 g of erythromycin rehydrate (Sigma-Aldrich) in 0.5 ml of 100% ethanol. Diluted stock solution (2000 µg/ml) of erythromycin was prepared prior to each MIC assay. MIC assay was performed on a 96 well plate (Falcon) and serial dilutions were used to achieve a range of concentrations that each differed by two-fold; 100 µl of diluted stock was placed in the first column and was diluted with 100 µl of LB broth. Ten µl of diluted cell cultures was placed into each well except for the last column which was used as a negative control. A positive control with no antibiotics added was also used. Each sample was tested in quadruplicate to ensure consistency in our results. Plates were placed in the 37˚C incubator for 20 hours and the MIC was measured. The MIC assay was read qualitatively by eye for the presence or absence of growth. We also quantitatively measured the plate using a Spectronic 20+ spectrophotometer, although it is important to note that an MIC assay is typically read by eye and is qualitative.

RESULTS

Confirmation of *wzb* knockout strain CWG344 using polymerase chain reaction. In order to confirm the strain CWG344 was in fact the *wzb* mutant, we used PCR to amplify the *wzy* cassette in the following strains: E69, CWG655Δ, CWG281, CWG343, and CWG344. The EB6 and EB7 primers used in this experiment were originally obtained from Reid and Whitfield by Su et al. and they flank the entire *wzy* cassette, which is approximately 4200 bp. Gel electrophoresis was used to resolve the PCR

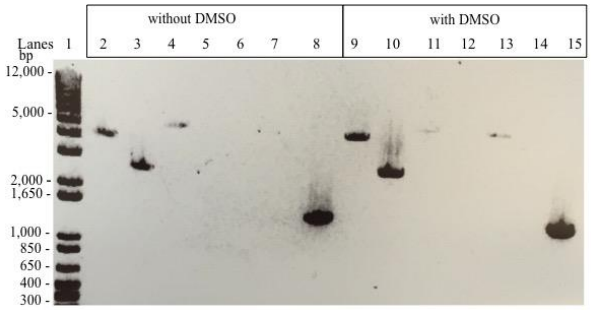

FIG 1 PCR amplification of *wzy* cassette in E69, CWG281, CWG343, CWG344, and CWG 655 with and without 1% DMSO. The *wzy* cassette was amplified using PCR in the wild type E69, CWG281, CWG343, CWG344, and CWG655. A 4200 bp band was expected for E69 whereas the amplified region in CWG281 should be larger than wild type and CWG655 should be smaller. Lanes 2-8 contained PCR reactions as outlined by Su et al. and described below, while lanes 9-15 contained the exact same PCR reactions in sequential order with an additional 1% DMSO. Lane 1: 1 Kb Plus DNA ladder. Lane 2 and 9: E69; Lane 3 and 10: CWG655; Lane 4 and 11: CWG281; Lane 5 and 12: CWG343; Lane 6 and 13: CWG344; Lane 7 and 14: negative control with sterile distilled water; Lane 8 and 15: positive control with E69 and primers against OmpC.

products. As shown in Fig. 1, lanes 2-8 showed PCR reactions without 1% DMSO and lanes 9-15 showed the exact same PCR reactions with 1% DMSO. Lanes 7 and 14 showed the negative control and lanes 8 and 15 showed the positive control, which used primers against ompC. We see a band as expected around 4200 bp in lanes 2 and 9, which is the size of the *wzy* cassette in the wild type E69 (4). Lanes 3 and 10 also showed the expected bands for CWG655Δ, which should be less than 4200 bp since the *wzy* cassette has been replaced for a gentamicin resistance cassette which is 944 bp (4). Lanes 4 and 11 also showed that CWG281 has a band slightly larger than the wild type's, which is consistent with Su et al. Lanes 5 and 12 should have had bands larger than 4200 bp for CWG343, since kanamycin and spectinomycin resistance cassettes have been inserted in *wza* and *wzb* (3). However, these bands were missing. Lanes 6 and 13 should have shown a band larger than 4200 bp since CWG344 has a kanamycin resistance cassette inserted to inactive *wzb*, and our results were consistent with this. Although a band was missing for CWG344 in lane 6, the band was visible when supplemented with 1% DMSO in lane 13. Our results suggest that our strain of interest, CWG344 is the *wzb* mutant strain.

Deletion of *wza* is sufficient to confer resistance to erythromycin in solid media, but not in a liquid media. In order to confirm that the deletion of *wza* in *E. coli* is sufficient to confer resistance to erythromycin as suggested by Su et al., we tested the erythromycin sensitivity of a single *wza* knockout, CWG281, a *wza* and *wzb* double knockout, CWG343, and a *wza*, *wzb* and *wzc* triple knockout, CWG655Δ, and compared these to the wild type E69. We used a modified version of the Kirby-Bauer

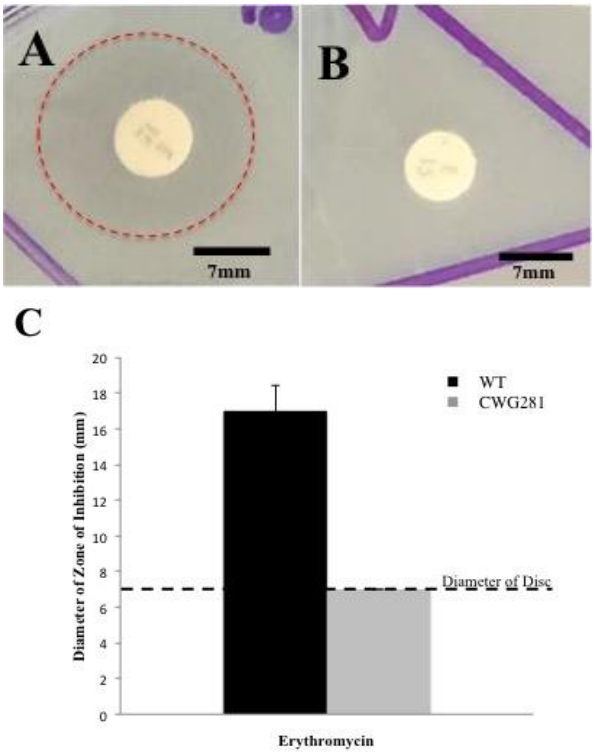

FIG 2 Susceptibility of WT (E69) and CWG281 to erythromycin via disc diffusion assay. Example for the zone of inhibition seen on the disc diffusion assay of E69 (A) demonstrates the susceptibility of these strains to erythromycin, as outlined by the red dashed line. The absence of a zone of inhibition on the disc diffusion assay of CWG281 (B) indicates its resistance to erythromycin. Scale bars = 7 mm. (C) Bar graph summarizing results of the disc diffusion assay for WT (E69) and CWG281. The diameter of the zone of inhibition was measured by going through the center of the disc to the opposite end of the zone. The average of the triplicate antibiotic discs is shown with error bars representing standard deviation on the mean of the triplicates. Erythromycin discs of 7 mm in diameter were used on cells plated on LB agar for this assay. An increase in susceptibility to erythromycin is seen by an increased diameter of the zone of inhibition.

antibiotic disc diffusion assay as reported by Botros et al., who previously reported increased erythromycin resistance of strain CWG655Δ (5). The average zone of inhibition of E69 was 17 mm (Fig. 2a, c), while CWG281 had no observable zone of inhibition (Fig. 2b, c). CWG655Δ and CWG343 also lacked an observable zone of inhibition (S. Fig. 1b, 1c, 2b, and 2c). This resistant phenotype of CWG655Δ and CWG281 is consistent with phenotypes observed by Su et al., and these results confirmed that deletion of *wza* is sufficient to confer resistance to erythromycin.

In addition to the disc diffusion assay, we also performed an MIC broth dilution assay to determine relative sensitivity to erythromycin. Both CWG655Δ and CWG343 exhibited a more resistant phenotype against erythromycin with higher MIC values in comparison to the wild type E69, consistent with the observations made in the disc diffusion assay

Strain	Erythromycin MIC (µg/ml)
E69 (WT)	100
CWG655Δ (Δ*wzabc*)	200
CWG343 (Δ*wzab*)	125
CWG281 (Δ*wza*)	100
CWG344 (Δ*wzb*)	100

TABLE 1 Minimum inhibitory concentrations of *Escherichia coli* E69, CWG655Δ, CWG281, CWG343, and CWG344 against erythromycin. Stock solution of erythromycin (2000 mg/ml) was prepared in 100% ethanol and further dilutions were made with LB media. Plates were incubated at 37°C for 20 hours and quantitatively measured using Epoch Microplate Spectrophotometer (Biotek). E69 is the wildtype strain that has the complete *wzy* gene cassette present. CWG655Δ is a knockout strain of the entire *wzy* gene cassette. CWG343 is the *wza* and *wzb* double knockout strain. CWG281 is a single deletion strain of *wza*, whereas CWG344 is a single deletion strain of *wzb*.

(Table 1). The resistance was more apparent in CWG655Δ than CWG343 as it had an MIC that was two-fold larger than the MIC of E69. However, the MIC assay indicated that CWG281 was as susceptible to erythromycin as the wild type and was not consistent with the observation in the disc diffusion assay; the MIC of CWG281 was 100 µg/ml, which is equal to the MIC of E69 (Table 1).

Deletion of *wzb* in *E. coli* does not confer resistance to erythromycin. After confirming that deletion of *wza* was sufficient to confer resistance to erythromycin we asked whether *wzb* also plays a role in the observed erythromycin resistance of CWG343 and CWG655Δ. To test this, we used strains E69, CWG281, CWG 343, CWG344, and CWG655Δ in disc diffusion assays according to the modified Kirby-Bauer method and included Whatman paper discs as a negative control. CWG344, the *wzb* mutant, showed a visible zone of clearance with an average diameter of 17.3 mm (Fig. 3b, c) and was comparable to the zone of clearance seen in E69, which had an average diameter of 17 mm (Fig. 3a, c). All strains bearing *wza* mutations, CWG281, CWG343 and CWG655Δ, showed resistance to erythromycin, as indicated by the absence of a zone of inhibition around the erythromycin disc. Furthermore, CWG344 had a minimum inhibitory concentration of 100 µg/ml against erythromycin, which is equivalent to that of the wild type E69 (Table 1). These results allow us to confirm that deletion of *wza* is sufficient to confer resistance to erythromycin and that *wzb* does not play a role in this observed resistance.

DISCUSSION

In this study, the role of *wzb* in capsule-independent resistance of *E. coli* K30 against erythromycin was examined. Previously, Botros et al. reported that CWG655Δ, a complete deletion mutant of the *wzy* group I capsule biosynthesis system, is resistant to erythromycin compared to the wildtype, *E. coli* E69 (5). Another group, Su et al., suggested that the deletion of *wza* is sufficient to induce this erythromycin-resistant phenotype (6). Although it is clear that *wza* plays a role in this observation, the role of *wzb* has not been

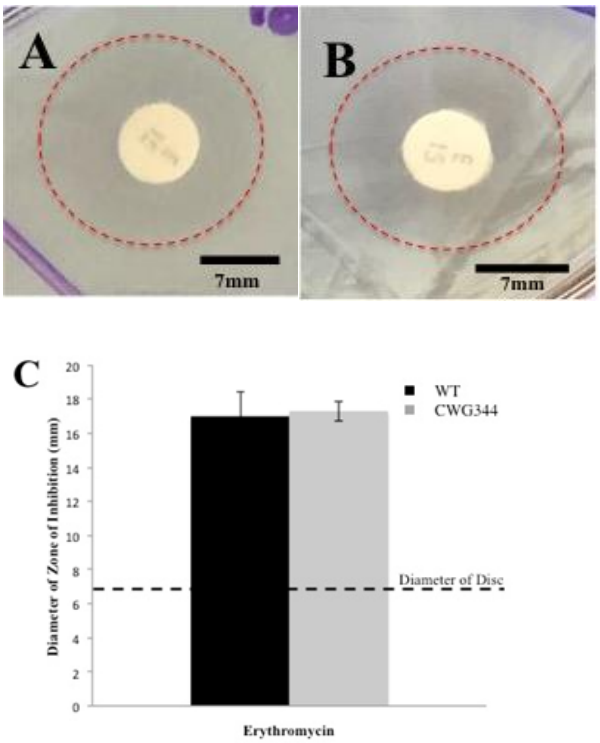

FIG 3 Susceptibility of WT (E69) and CWG344 to erythromycin via disc diffusion assay. Example for the zone of inhibition seen on the disc diffusion assay of E69 (A) demonstrates the susceptibility of this strain to erythromycin, as outlined by the red dashed line. The zone of inhibition seen on the disc diffusion assay of CWG344 (B) demonstrates the susceptibility of this strain to erythromycin, as outlined by the red dashed line. Scale bars = 7 mm. (C) Bar graph summarizing results of the disc diffusion assay for WT (E69) and CWG344. The diameter of the zone of inhibition was measured by going through the center of the disc to the opposite end of the zone. The average of the triplicate antibiotic discs is shown with error bars representing standard deviation on the mean of the triplicates. Erythromycin discs 7 mm in diameter were used on cells plated on LB agar for this assay. An increase in susceptibility to erythromycin is seen by an increased diameter of zone of inhibition.

previously elucidated. We began our study by validating the strains using PCR and replicating the experiments. Our PCR results were consistent with the findings of Su et al. and confirmed the expected genotypes of the strains used. E69 showed a band that correlates to the presence of the *wzy* cassette. The PCR product of CWG655Δ strain produced a band that corresponds to the gentamicin resistance cassette (Fig. 1). In addition, CWG281 and CWG344 showed bands that matched the expected sizes. However, there was an absence of a band for the CWG343 strain. Addition of 1% DMSO also enhanced the quality of the PCR reactions, indicated by the difference in clarity and band intensity between the PCR reactions with and without the use of DMSO (Fig. 1). DMSO is a known enhancer of PCR reactions as it reduces the secondary structures that interfere with the replication process (10).

From the disc diffusion assay we saw a clear zone of inhibition when CWG344 was grown in the presence of erythromycin discs on regular LB agar plates. The knockout strain also had the same minimum inhibitory concentration of erythromycin as the wildtype E69 strain, at 100 micrograms per milliliter. None of the results suggest that a knockout in *wzb* increases the resistance of the bacteria to erythromycin. All indications point to CWG344 having the same susceptibility to erythromycin as the wildtype strain. This result refutes our original hypothesis of the Wzb phosphatase function playing a role in dephosphorylating inactivated macrolides. However, disc diffusion results from the *wza* mutant, CWG281, were in line with what Su et al. had observed. In other words, knock-out of *wza* did increase erythromycin resistance of the bacteria. This is true for all knock-outs of *wza*, including the triple knockout of *wzy* in CWG655Δ, as well as the double knock-out of *wza* and *wzb* in CWG343. The mechanism proposed by Su et al. suggested that Wza plays a role in antibiotic susceptibility by acting as a channel for the antibiotic to enter the cell. Although this could be a possible mechanism that explains our results, we are unable to conclude this from our data. Although we are able to rule out *wzb* playing a role in the resistance towards erythromycin, from our results we are unable to rule out *wzc* in order to conclude that only *wza* is involved.

Further work investigating *wzc* is required to see if *wza* is the only gene out of the cassette that is responsible for the observed resistant phenotype. Wzc has been shown to help form the translocation system required for transporting capsule out of the cell (4). There is a possibility that this translocation system can also be used by antibiotics to enter the cell, which is why we cannot completely rule out Wzc playing a role in resistance towards macrolides. However, from our data we are not able to determine whether it is the genes of the *wzy* cassette that are upregulating or downregulating another resistance gene or if it is the proteins themselves that are mechanistically involved in resistance. Further experiments involving mutating the functions of the *wzy* proteins, as opposed to deleting these genes, will help to determine if it is indeed the proteins themselves that are mechanistically responsible for resistance or not.

We conducted the minimum inhibitory concentration assay to confirm the results that were observed in the disc diffusion assay and expected CWG655Δ, CWG281, and CWG343 to have higher MIC values relative to the wildtype control, E69. Also, since CWG344 displayed a visible zone of inhibition on the disc diffusion assay that was similar in size to E69, we expected these strains to have similar sensitivities to erythromycin in the disc diffusion assay. Interestingly, while CWG344 did have the same MIC as E69 as expected (Table 1), and CWG655Δ and CWG343 showed consistent resistance between the two assays, such was not the case for CWG281. The observed resistance towards erythromycin that was seen with CWG281 on the disc diffusion assay was not seen in the MIC assay, and these results are consistent with those seen by Su et al. While we first speculated that the high ethanol concentration in the MIC protocol by Su et al. was playing a role in the discrepancies observed between the two assays, these results were reproduced even after reducing the ethanol concentration in the protocol. We speculate whether the difference in results between the two assays is due to the differences of capsule growth in solid versus liquid media, which has previously been seen in studies involving *Staphylococcus aureus* by Lee et al. (11). In particular, *S. aureus* was seen to produce higher amounts of type 8 cellular polysaccharide when grown on a surface media compared to liquid media (11). As such, if the solid media used in our disc diffusion assay allowed for greater production of capsule compared to the liquid LB broth used in the MIC, it may have been assisting in the higher levels of antibiotic resistance that were observed on the solid media compared to the liquid media.

Overall, we conclude that the deletion of *wzb* alone is not sufficient to confer antibiotic resistance to erythromycin. This restricts the capsule-independent antibiotic resistance to come either from *wza* alone or both from *wza* and *wzc*.

FUTURE DIRECTIONS

The increased macrolide resistance of CWG655Δ, which was previously reported by Botros et al., could have been due to the inactivation of either *wza*, *wzb*, or *wzc*, or a combination of these genes (5). While our results suggest that *wzb* is not required for the observed antibiotic resistance, previous studies by Su et al. have shown that *wza* is necessary and sufficient. A future study investigating the effects of a single knockout of *wzc* is required to fully elucidate the role of Wzc in macrolide resistance. This final piece of evidence will complete the existing interactions between the different players of group I capsular genes investigated that could be involved in the resistance of CWG655Δ towards erythromycin.

Another direction that would offer valuable insight into the role of the *wzy* cassette in antibiotic resistance would be to investigate the sensitivity of all the strains used in this study to additional antibiotics. Botros et al. have previously reported that deletion of the entire *wzy* cassette in CWG655Δ conferred resistance to other macrolides, including clarithromycin and roxithromycin, in addition to tetracycline (5). It would be valuable to investigate the role of the single knockout of *wza* and *wzb* to these different antibiotics in order to observe whether the results of these single knockouts that we have obtained in our study against erythromycin are consistent with other antibiotics. These results would assist in further clarifying the role of the *wzy* cassette in antibiotic resistance.

It would also be interesting to test different classes of antibiotics, as studies on the genes of the *wzy* cassette have until this point focused on macrolides. It would be a useful investigation to perform the MIC and disc diffusion assays of each *E. coli* K30 knockout mutant against different classes of antibiotics to see whether a different knockout, or combinations of knockouts, of the *wzy* gene cassette would result in resistance that differs among different classes of antibiotics.

Repeating the assays from our research with the *wza* and *wzc* double knockout, as well as the *wzb* and *wzc* double knockout, may be worthwhile, as these are the remaining double knockout combinations of the *wzy* gene cassette. It may be interesting to see if the antibiotic resistance phenotypes are consistent among all combinations of knockouts, as these results could elucidate possible interactions between the expressed proteins.

Finally, an experiment to investigate whether or not the pore function of *wza* is necessary for its effects on antibiotic resistance would be useful. This experiment could be done by using a functional mutant of *wza*, as well as changing the hydrophobicity of the pore protein. As mentioned in the discussion, this experiment will help to clarify whether it is the actual protein that is mechanistically involved in the resistance, or if it is the genes of the cassette that are downregulating or upregulating another antibiotic component within the cell.

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