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Deletion of the Group 1 Capsular Gene *wza* in *Escherichia coli* E69 Confers Resistance to the Antibiotic Erythromycin on Solid Media but not in Liquid Media

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It has been suggested that the presence of a capsule influences the level of antibiotic resistance of a bacterium, although there have been contradicting studies in the literature. Previous research demonstrated that the deletion of three key group 1 capsular biosynthesis genes (wza, wzb and wzc), components of the Wzy-dependent polymerisation system, from Escherichia coli E69 leads to resistance to macrolide antibiotics. It is not known if this is the effect of deleting a single gene or the entire gene cluster. Wza forms a channel in the outer membrane suggesting that macrolides may diffuse into the cell through the Wza pore. In this study, we asked whether a deletion of the wza gene results in the macrolide resistant phenotype observed in the Wzy triple deletion strain. Antibiotic disc diffusion and microdilution assays were performed on an E. coli wza knockout strain and a Wzy triple gene deletion (wza, wzb, wzc) E. coli strain. Both strains exhibited resistance to erythromycin in antibiotic disc diffusion assays compared to wild type E. coli strain E69. In contrast, the wza knockout E. coli strain was more sensitive to erythromycin than the wild type E. coli stain E69 and the Wzy triple deletion mutant in microdilution assays. We conclude that the deletion of wza is sufficient to induce resistance toward erythromycin on solid media but not on liquid media.

Capsular polysaccharides surround the cell surface of many bacterial species providing protection from environmental stresses, desiccation, and potentially antibiotics (1). How the capsule impacts the antibiotic resistance capabilities of *Escherichia coli* is not well understood. Two studies have reported capsule-dependent resistance to kanamycin (2, 3) and streptomycin (2) in *E. coli*. Another study found that capsule does not confer resistance against streptomycin (4). Drayson *et al.* suggested that capsule-dependent antibiotic resistance is not universal and may depend on the antibiotic class (5).

Macrolides are a class of antibiotics characterized by macrocyclic lactone rings (6) that inhibit protein synthesis by binding to the 50S ribosomal subunit (6). Strains of *E. coli* bearing mutations in the structure of the outer membrane (OM) show sensitivity towards erythromycin compared to wild-type *E. coli* strains (7). It has been proposed that the OM of Gram negative bacteria may prevent uptake of macrolides resulting in increased resistance (7).

In *E. coli* strain E69, the Wzy-polymerization system synthesizes K30 (group 1) capsule (8). The Wzy-polymerization system includes 3 key genes: *wza, wzb,* and *wzc*. Wza is an outer membrane lipoprotein that forms a channel in the outer membrane through which capsule polysaccharide subunits are translocated to the cell surface (9). Wzc is an integral inner membrane protein involved in the polymerization of capsule subunits through tyrosine kinase activity (10). The role of Wzc has been theorized to be a determinant of capsular polymer chain length or a coordinator of multi-enzyme complexes for capsular polysaccharide synthesis (8)The role of Wzc has been theorized to be a determinant of capsular polymer chain length or a coordinator of multienzyme complexes for capsular polysaccharide synthesis (8). Wzb is a cytoplasmic phosphatase of Wzc and is required for a wild-type capsule phenotype (8). These genes appear to be essential for capsule expression in *E. coli* (8). Botros *et al.* demonstrated that the deletion of these three genes resulted in resistance to erythromycin, a macrolide antibiotic (11).

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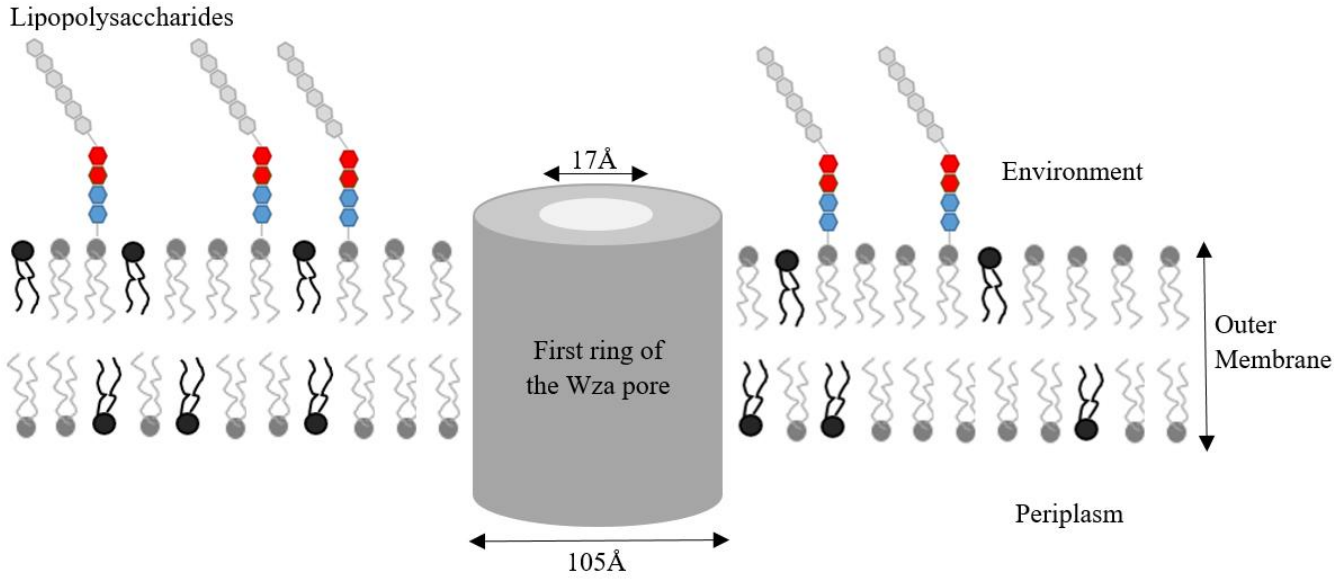

FIG. 1 Schematic diagram of the segment of the Wza pore crossing the outer membrane (14). The ring shown is a helical barrel which forms the neck of the Wza pore crossing the outer membrane. At the open end, the internal diameter is 17Å wide, large enough for erythromycin to fit through. Subsequently, there are three larger rings crossing the periplasm (not shown in figure) which complete the structure.

Entry of macrolides likely occurs through passive diffusion across the outer membrane (12). It is possible that the Wza pore plays a role in the observed resistance of the triple gene deletion due to its location in the outer membrane and structure as a molecular conduit for capsule subunits translocation (13). Wza has an octameric structure described as an amphora consisting of four rings (Fig. 1) (14). Using the length of a C-C bond, which is 1.33Å (15), we estimate that erythromycin is approximately 11.97Å horizontally and 10.64Å vertically (Fig. 2). Based on this estimate, it is possible that erythromycin diffuses into *E. coli* through the Wza pore as it is able to fit through the smallest ring, which has a pore opening of 17Å in diameter (Fig. 1). It is important to note that the hydrophobic properties of erythromycin may also affect its movement across the hydrophilic membrane (16). In addition to a triple capsular gene deletion strain, a *wza* gene deletion strain has also been developed by Reid and Whitfield (8).

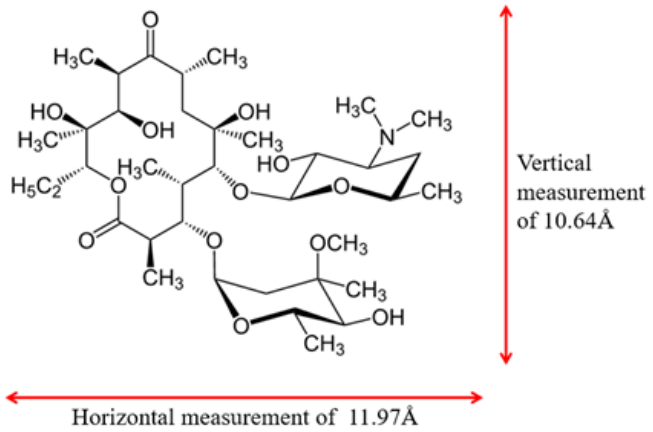

FIG. 2 Representation of how the size of erythromycin was measured. The measurements were based on the size of C-C bond (1.33Å). One C-C bond in the structure was measured with a ruler, and the measurement was used as a baseline for the size of one C-C bond. The structure was then measured horizontally and vertically, and then converted into Å using the ruler measured C-C bond size.

Since the Wza pore is a potential first point of contact for erythromycin as it encounters the cell, the pore may play a part in its subsequent entry explaining how it is able to enter. We hypothesized that deletion of *wza* in *E. coli* E69 is sufficient to confer resistance to macrolides as observed in the triple gene knockout deletion. We hypothesized that deletion of wza in E. coli E69 is sufficient to confer resistance to erythromycin as observed in the triple gene deletion given the size of the Wza channel. In our study, it was observed that the *wza* gene does alter the susceptibility of *E. coli* strain E69 to erythromycin and is sufficient to confer resistance to this specific macrolide antibiotic. Interestingly, the result was only observed in assays done on solid media but not in assays done in liquid media. It is possible that the mechanism of action or uptake of erythromycin varies based on the nature of the growth media.

MATERIALS AND METHODS

Bacterial strains, preparation of media and growth conditions. *E. coli* wild-type (WT) E69 (serotype: O9a:K30:H12) and CWG655 [$wza_{22\ min}$::*aadA* Δ(*wza-wzb-wzc*)$_{K30}$::*aphA3*] were obtained from the MICB 447 laboratory stock from the Department of Microbiology and Immunology at the University of British Columbia (UBC). CWG281 ($wza_{22\ min}$::*aadA*wza_{K30}::*aacC1*) was obtained from the laboratory of Dr. Chris Whitfield from the Department of Molecular and Cellular Biology at the University of Guelph. *E. coli* E69 is the isogenic parental strain of CWG655 and CWG281. Most *E. coli* isolates, such as E69, contain two chromosomal copies of

the *wza-wzb-wzc* cluster. One copy is in the *cps* locus and the other copy is at a position that corresponds to 22 min on the *E. coli* K-12 linkage map (8). For both strains, a polar *aadA* insertion in the 22-min copy of *wza* eliminated the expression of the 22-min copy of the *wza-wzb-wzc* cluster (8). For CWG655, the entire gene cluster in the *cps* locus was replaced by a kanamycin resistance cassette via homologous recombination. For CWG281, the *wza* gene was inactivated using a gentamycin resistance cassette insertion within the gene (9). As such, CWG655 has all three genes knocked out while CW281 only has the *wza* gene knocked out. All growth experiments were performed at 37°C. Liquid cultures were incubated in an incubator shaker, and plates were incubated in an incubator. Bacterial 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) and as needed, were plated on 1.5% agar LB media plates.

Disc diffusion assays. Using the Kirby-Bauer method modified by Botros *et al.*, antibiotic disc diffusion assays were performed on the three strains (11). A colony of each strain was inoculated in 5ml of LB media and were grown overnight at 37°C. The optical densities were measured at 660nm using a Spectronic 20+ spectrophotometer (Pharmacia Biotech) the following day. The cultures were diluted with sterile LB broth to ~0.8 OD unit to normalize the OD of the three cultures. 100µL of each diluted culture was then spread onto LB agar plates and allowed to dry. Erythromycin antibiotic discs (7mm diameter) obtained from the Department of Microbiology and Immunology at UBC were placed on the plates using sterile forceps. Blank discs were also placed on the LB plates to act as a negative control. Each plate contained three erythromycin discs and one blank disc, each strain was plated in triplicate. The plates were incubated for 19 hours at 37°C. The diameters of the zone of inhibitions were measured in millimetres. A clearance around the disc indicates susceptibility to erythromycin, where an increase in the diameter indicates increased susceptibility. Conversely, a decrease in size of the zone of clearance indicates increased resistance.

Minimal inhibition concentration determination by microdilution assays. A broth microdilution minimal inhibitory concentration (MIC) assay was performed (17). A colony of each strain was inoculated in 5ml of liquid LB broth and grown overnight at 37°C. OD_{660nm} readings were measured the following day using a Spectronic 20+ spectrometer. Using LB broth, the overnight cultures were then diluted down to 0.0001OD units, which corresponds to 10^5cells/ml. A 10mg/ml stock solution of erythromycin was made by adding 0.05g of erythromycin rehydrate (Sigma-Aldrich) to 5ml of 100% ethanol. Prior to the assay, the stock solution was diluted down to 4000µg/ml with LB broth. 5 rows of a 96-well plate was filled with 100µl of sterile LB broth. The first column of the plate was filled with 100µl of the diluted erythromycin (4000µg/ml) and mixed by pipetting up and down 10 times. 100µl of the solution in the first column was then transferred to the second column to create a two-fold dilution. This was done for each column up to column 10, where 100µl was discarded. The concentrations were two-fold differences starting from 4000µg/ml and ending with 7.8µg/ml. 5µl of the 10^5cells/ml cultures were then added to every well with solution except for column 12. Column 11 contained the bacteria to serve as a positive control and column 12 contained LB broth to serve as a negative control. The assay was done in replicates of 5 for each strain. Plates were placed in the 37°C incubator for 19 hours and were inspected visually the following day to identify the lowest concentration needed to inhibit the growth of the bacteria based on visual growth in the media.

TABLE 1 Primer sequences and features used in the amplification of capsule biosynthesis cluster in WT, CWG 281, and CWG655.

Primer	Primer sequence (5′ to 3′)	Melting temperature (°C)
Designed forward	cgcttgaatacttcactgtggt	55.2
Designed reverse	tttgatcttgtggaactctctg	52.5
EB6	ggtcagggatccaacagtctg	57.3
EB7	tcgcggatccaattgttacga	56.6

PCR primer design for amplification of *wza*, *wzb*, and *wzc* region. Primers were designed by first obtaining the sequence of the *E. coli* K30 capsule biosynthesis cluster from NCBI, accession number AF104912 from GenBank. A sequence of 22 nucleotides was chosen approximately 200 base pairs (bp) before the start of the *wza* gene, and 200 bp after the end of the *wzc* gene. They were determined to have similar melting temperatures according to IDT. Furthermore, primers were taken from Reid and Whitfield (EB6 and EB7), which amplify the region inclusive of *wza*, *wzb*, *wzc*, and *wbaP* (8). 3 nucleotides were removed from the 5′ end of EB6 to reduce the melting temperature. Both sets of primers were used to confirm the region of deletion in CWG655 and CWG281 by PCR. All primers used in this study are listed in Table 1.

PCR amplification of *wza*, *wzb*, and *wzc* region. Lyophilized primers (25nmol) acquired from the Nucleic Acid Protein Service Unit at the University of British Columbia, Vancouver campus. They were resuspended in 2.5ml of sterile distilled water to reach primer concentrations of 50µM. As specified by the Invitrogen™ Platinum™ *Taq* Polymerase procedure provided by ThermoFisher Scientific, each PCR reaction mix was made from: 5µl of 10X PCR Buffer (-$MgCl_2$), 1.5µl of 50 mM $MgCl_2$, 1µl 10mM dNTP mix, 1µl 10µM forward primer, 1µl 10µM reverse primer, and either 1µl template DNA (pGLO GFP) or a colony of the strains (WT, CWG281, CWG655), 0.2µl of *Taq* DNA polymerase, and either 33.3µl or 38.3µl of sterile distilled water, for a total volume of 50µl. The PCR conditions for all the reactions were 94°C for 120 seconds for the initial cycle, followed by 35 cycles consisting of 30 seconds of 94°C, 30 seconds of 50°C, and 4.83 minutes at 72°C with a final cycle for extension of 10 minutes of 72°C.

Gel electrophoresis of PCR products to confirm presence or absence of *wza*, *wzb*, *wzc* region. In order to confirm that the strains used were of the correct genotype, the *wza, wzb* and *wzc* gene cluster was amplified by PCR and visualized on a 1% agarose made from 0.2g of agarose in 20ml of SYBR safe DNA dye in 0.5x TBE. The gel was run at 120V for 1 hour. 0.3µl of 10X DNA loading dye was loaded with each 2.7µl sample of PCR product. 2.7µl of 1 Kb Plus DNA Ladder was loaded into the gel with 0.3µl of 10X DNA loading dye. The gel was visualized using the Alpha Innotech AlphaImager.

RESULTS

***E. coli* strain CWG655 bearing triple gene deletion (*wza*, *wzb*, *wzc*) show increased erythromycin resistance.** Using a modified version of the Kirby-Bauer antibiotic disc diffusion assay, Botros *et al.* demonstrated that growth of wild type (WT) *E. coli* strain E69 is inhibited by erythromycin while *E. coli* strain CWG655 bearing a Wza, Wzb, Wzc deletion was observed to be resistant (11). Botros

et al. performed the assay using paper discs impregnated with erythromycin. *E. coli* strains were spread as a thin film on solid LB agar plates and the antibiotic disc was placed on the surface. The plates were then incubated overnight at 37°C. A WT *E. coli* strain E69 was tested side by side with *E. coli* strain CWG655 (triple deletion of *wza*, *wzb*, and *wzc*) in order to confirm susceptibility to erythromycin observed by Botros *et al*. (11). The average zone of clearance around the erythromycin disc was 14mm in diameter for the WT strain while the zone observed for the CWG655 strain was 9.4mm (Fig. 3A, C, Fig. 4A, C). These results suggest that both the WT and CWG655 strain are susceptible to erythromycin but CWG655 is more resistant as indicated by the smaller clearance zone. These data are consistent with the results reported by Botros *et al*. (11).

Deletion of *wza* in *E. coli* strain E69 is sufficient to confer resistance to erythromycin. Having confirmed susceptibility toward erythromycin in WT *E. coli* strain E69 and a degree of resistance toward erythromycin in the triple deletion *E. coli* strain CWG655, we asked whether deletion of the *wza* gene is sufficient for resistance to erythromycin observed in the triple deletion strain CWG655. Disc diffusion assays using erythromycin antibiotic discs were performed using CWG281 (which has *wza* inactivated), WT *E. coli* strain E69 and the triple deletion strain CWG655. There were no clearance zones around the erythromycin discs for all of the assays done with strain CWG281 (Fig. 4B, C). 7mm sterile paper discs were placed on the LB agar plates alongside the erythromycin discs, to serve as a negative control. As expected, there was no clearance zone around these discs. The average clearance zone size for the *wza* deletion strains were 7mm, the same as the negative control discs, which demonstrates that CWG281 bearing a single gene knockout of *wza* is resistant to erythromycin. This result was comparable to the disc diffusion assays of the triple gene deletion knockout (CWG655) in which the zones of clearance was observed to be 9.4mm in diameter. We conclude that deletion of *wza* alone is sufficient for erythromycin resistance in a disc diffusion assay performed on solid LB agar media.

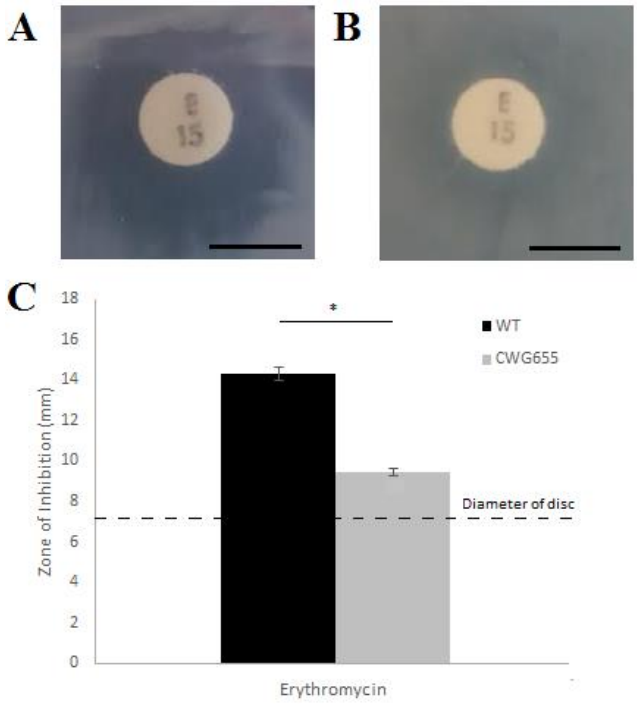

FIG. 3 Susceptibility of WT and CWG655 strains to erythromycin via disc diffusion assay. (A, B) Disc diffusion assays show that WT is susceptible to erythromycin, indicated by a zone of clearance around the erythromycin disc. CWG655 is shown to be less susceptible to erythromycin, as indicated by the reduced zone of clearance around the disc. Scale bars = 7mm. **(C)** Bar graph representatives of (A, B). Mean value from triplicates. Error bars represent standard error of the mean. Disc diffusion assays were incubated for 19 hours at 37°C on LB agar plates. Zone of inhibition refers to zone of clearance, and the diameter was measured. Increase in diameter of zone of clearance indicates increased susceptibility, whereas a decrease in diameter indicates increased resistance. *indicates p-value <0.05.

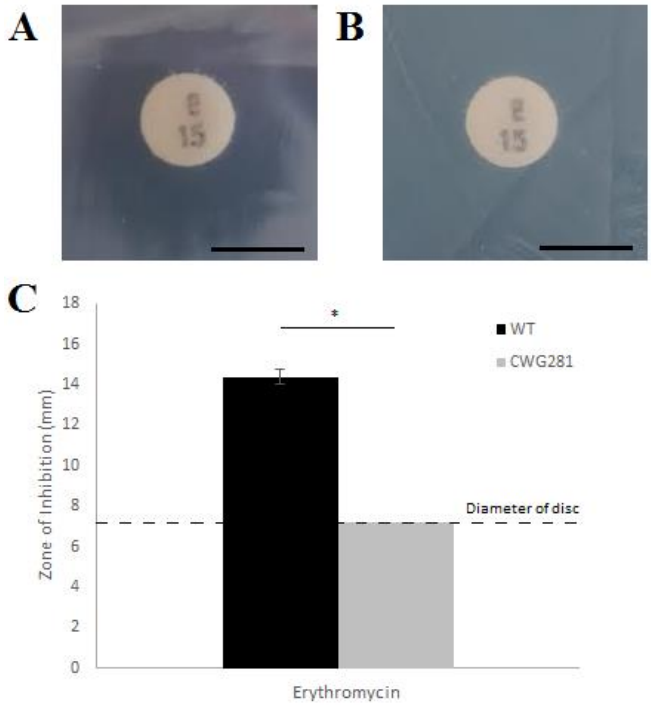

FIG. 4 Susceptibility of WT and CWG281 strains to erythromycin via disc diffusion assay. (A, B) Disc diffusion assays show that WT is susceptible to erythromycin, indicated by a zone of clearance around the erythromycin disc. CWG281 is shown to be resistant, as indicated by the lack of any zone of clearance around the disc. Scale bars = 7mm. **(C)** Bar graph representatives of (A, B). Mean value from triplicates. Error bars represent standard error of the mean. Disc diffusion assays were incubated for 19 hours at 37°C on LB agar plates. Zone of inhibition refers to zone of clearance, and the diameter was measured. Increase in diameter of zone of clearance indicates increased susceptibility, whereas a decrease in diameter indicates increased resistance. *indicates p-value <0.05.

Minimum inhibitory concentration of erythromycin performed in liquid media was lower for strain CWG281 than WT *E. coli* strain E69 and triple deletion mutant CWG655. To further investigate the erythromycin resistant phenotype associated with the *wza* inactivation, we used a microdilution assay to evaluate the minimum inhibitory concentration (MIC) of erythromycin that would inhibit growth of WT, CWG281 and CWG655. Each strain was subjected to erythromycin serially diluted in liquid LB from 4000-7.8µg/ml in a 96-well plate. Plated were incubated for 20 hours at 37°C to assess growth in each condition. The

MIC of erythromycin for WT and CWG655 were determined to be 250µg/ml while CWG281 was 125µg/ml (Table 2). This was observed in all 5 replicates, in two different trials with no variation between the MICs (Table 2). These results are contrary to our disc diffusion assays, suggesting that the strains display different sensitivities when exposed to erythromycin on liquid versus solid media.

Confirmation of knockout strains CWG281 and CWG655 using polymerase chain reaction. In order to ensure that strains CWG281 and CWG655 were indeed the correct mutants, we amplified the K30 capsule biosynthesis gene cluster in WT, CWG281, and CWG655. PCR was performed using primers EB6 and EB7 obtained from Reid and Whitfield as well as primers designed in this study flanking the gene cluster (8). PCR products were resolved by agarose gel electrophoresis (Fig. 5). Lanes 2 and 3 show positive and negative PCR control reactions. According to accession number AF104912 from GenBank we expected the *wza*, *wzb*, and *wzc* region in wild type *E. coli* strain E69 to be approximately 4200 bp. Lanes 4 and 7, containing PCR products amplified using WT *E. coli* strain E69 DNA as a template showed a band of 4200 bp, consistent with our prediction. The knockout of *wza* in CWG281 was created by insertion of a 616 bp gentamicin resistance cassette into the gene (18), therefore the PCR product should be around 4816 bp (Fig. 5). In lanes 5 and 8, which contained the PCR product of CWG281, there is a band larger than 4200 bp, which is consistent with our prediction. The triple knockout in CWG655 was created by replacing the entire region with a 944 bp kanamycin resistance cassette through homologous recombination (18), thus the PCR product should be smaller than that of the WT (Fig. 5). Lane 9, containing the PCR product of CWG655, there is a band significantly smaller than 4200 bp.

TABLE 2 Minimum inhibitory concentrations of WT, CWG281 and CWG655 strains towards erythromycin, incubated in LB broth at 37°C for 20 hours.

Strain	MIC of Erythromycin (µg/ml)	
	Trial 1	Trial 2
WT	250	250
CWG281	125	125
CWG655	250	250

Lane 6, containing the PCR product of CWG655 using primers designed by us, should have had a similar band to lane 9. However, no band was observed, which could be due to our designed primers not being at the proper position to flank the whole region of deletion in CWG655. Taken together, these results suggest that each of the strains were of the expected genotype.

DISCUSSION

Erythromycin sensitivity assays were performed to determine if the deletion of *wza* in *E. coli* strain CWG281 is sufficient for erythromycin resistance, as observed in the *wza wzb wzc* triple deletion strain CWG655 (11). Our disc diffusion assays were consistent with the findings of Botros *et al.* and confirm that CWG655 bearing a triple deletion of gene *wza*, *wzb*, and *wzc* does indeed show resistance to erythromycin compared to wild type strain E69 (Fig. 3) (11).

We hypothesized that *wza* gene is involved in conferring sensitivity to erythromycin since it encodes for the Wza outer membrane channel. By analyzing the crystal structure of the Wza channel we postulated that it would form a pore able to accommodate passage of erythromycin across the outer membrane. Consistent with our hypothesis, CWG281 (a *wza* knockout strain) showed complete resistance in an erythromycin disc diffusion assay (Fig. 4A, C). It is tempting to speculate that the absence of the Wza channel eliminates the conduit through which erythromycin translocates. Our results are also consistent with previous studies showing increased susceptibility to macrolides when modifications to the OM are made that impact its permeability (7, 11). The results by Vaara *et al.* show that *E. coli* strains with outer membrane mutations that increase permeability are more sensitive to antibiotics compared to a WT strain (7). Vaara *et al*. used a microdilution method with liquid media to determine the MIC whereas we used solid media to determine the MIC. Previous studies done with mutations in the *wza* gene in *E. coli* strain K-12 indicate that it is not involved in resistance to streptomycin (19). This is consistent with our initial studies comparing *E. coli* strain K-12 and *E. coli* strain E69 in which we found the two strains differed in their susceptibility towards erythromycin.

We found that *E. coli* strain K-12 was resistant to erythromycin, with no visible clearance zone, while *E. coli* strain E69 had a clearance zone surrounding the erythromycin disc, suggesting that it is susceptible to erythromycin (Fig. S1, Fig. 3A). This is not surprising as various strains of *E. coli* show differing susceptibilities to antibiotics.

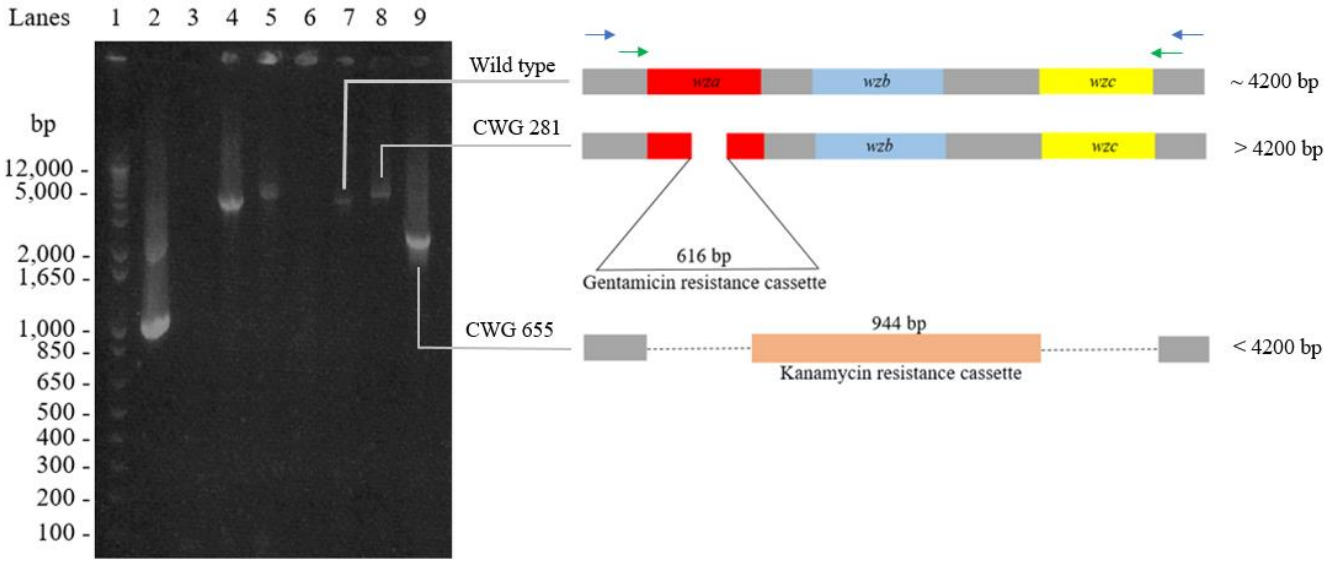

FIG. 5 PCR amplification of K30 capsule biosynthesis gene cluster in WT, CWG281, and CWG655. We used Whitfield's primers EB6 and EB7 (blue arrows) as well as our own designed primers (green arrows) to amplify the capsule biosynthesis cluster in the strains. The wild type region should be approximately 4200 bp. The CWG281 region should be larger than 4200 bp, due to the insertion of the 616 bp gentamicin resistance cassette within *wza*. The CWG655 region should be smaller than 4200 bp, due to the exchange of *wza*, *wzb*, and *wzc* with the 944 bp kanamycin resistance cassette. Lane 1: 1 Kb Plus DNA ladder. Lane 2: Positive control with primers and template that worked in a previous PCR. Lane 3: negative control with distilled water and our designed primers. Lane 4: *E. coli* E69 region amplified with designed primers. Lane 5: *E. coli* CWG281 region amplified with designed primers. Lane 6: *E. coli* CWG2655 region amplified with designed primers. Lane 7: *E. coli* E69 region amplified with EB6 and EB7. Lane 8: *E. coli* CWG281 region amplified with EB6 and EB7. Lane 9: *E. coli* CWG2655 region amplified with primers EB6 and EB7.

The results from the disc diffusion assays demonstrate that CWG281 (*wza* deletion) and CWG655 (*wza, wzb, wzc* deletion) strains are resistant to erythromycin compared to WT strain E69. Given these results, we expected CWG281 and CWG655 to have a higher MIC compared to the WT strain in broth dilution assays. Surprisingly, strain CWG281 (*wza* deletion) was more sensitive to erythromycin than the WT E69 and strain CWG655 *(wza, wzb, wzc* deletion) in broth dilution assays (Table 2). We verified the strains using PCR to confirm that the strains had not been switched. The result from the microdilution assay is consistent with studies where mutants with reduced outer membrane function were shown to be more sensitive to macrolides (7). This difference in phenotype of the *wza* deletion strain (CWG281) could be due to the use of different media forms in the assays. The various *E. coli* strains were grown on solid agar plates for the disc diffusion assays, but liquid media was used for the microdilution assay. It could be that CWG281 expresses different resistant mechanism in solid versus liquid media. For example, CWG281 may grow differently or express unique proteins in the two medias, which may have led to the contrasting resistance results. Due to time constrains, we were unable to study if there were differences in capsule production due to growth in different media forms, and if that affects their resistance towards erythromycin. Previous studies conducted with *E. coli* K-12 show discrepancies regarding antibiotic resistance dependency on capsule production (4, 5). It has been suggested that increased antibiotic resistance of strains due to the removal of capsule genes is not universal and may be different among the various antibiotic classes (5). This may explain the contradictions between the studies (2, 3, 4) and why antibiotic resistance has been observed to be either capsule dependent or independent (5).

In conclusion, we have shown that deletion of the *wza* gene is sufficient to confer resistance to erythromycin on solid media but not on liquid media. Our results suggest that the Wza pore may be an outer membrane portal through which erythromycin enters the cell. Erythromycin is smaller than the pore diameter of Wza, and could conceivably passively diffuse through the pore. However, the apparent antibiotic specificity of this resistance phenotype suggests defined molecular interactions between the macrolides and the Wza channel. Alternatively, the presence of Wza may be required for the stability of other proteins that may

impact macrolide activity or movement across the outer membrane (21).

FUTURE DIRECTIONS

Although our results indicate that *wza* is important for macrolide resistance we cannot exclude the possibility of *wzb* or *wzc* playing a role as well. Going forward, it would be interesting to examine the effect of *wzb* and *wzc* individually, to determine if they also contribute to macrolide resistance, or if *wza* alone is responsible for the observed phenotype. Other genes which stabilize the outer membrane, such as liposaccharides or porins (21), can also be studied to determine their effect on macrolide resistance.

Because the biosynthesis and assembly mechanism of the capsule in *E. coli* K30 is Wzy-dependent whereas *E. coli* K-12 is ABC transporter-dependent (22), it is possible the deletion of capsular biosynthesis genes would have different effects on antibiotic resistance in a K-12 strain. We observed that the K-12 strain BW25113 was resistant to macrolides using a disc diffusion assay even without any deletions of the capsular genes (Fig. S1). A possible future direction would be to use a microdilution assay to determine if the deletion of *wza* in the K-12 strain has any effect on macrolide susceptibility.

Finally, it would be useful to consider if deletion of *wza* has any effect on the resistance of other classes of antibiotics. It could either confer resistance or increase sensitivity based on how the antibiotics inhibit growth or enter the cell. We speculate that a *wza* deletion would increase resistance to antibiotics which enter the cell similarly to macrolides. Further investigation would provide insight on antibiotic resistance and possible methods of maximizing antibiotic effectivity. Our study focused on one antibiotic under the class macrolides, and further experiments could also be done with other macrolide antibiotics to determine if there are similar effects. In addition, we also have demonstrated that resistance to erythromycin differs in solid or liquid media. It would be interesting to see if changing media affects the susceptibility of *E. coli* to other antibiotics or if this is exclusive to erythromycin.

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