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Macrolide Structures Can Confer Differential Susceptibility in *Escherichia coli* K30 Deletions of Group 1 Capsule Assembly Genes

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The Wzy-dependent biosynthesis system in *Escherichia coli* K30 is responsible for the assembly and transport of extracellular capsule polysaccharides. The Wzy system has three key components: Wza, a transport channel located on the outer membrane; Wzc, an autokinase in the inner membrane coupled to Wza; and Wzb, a cytosolic phosphatase that regulates Wzc activity. Knock-outs of this assembly system have been observed to correlate with increased resistance to some macrolide antibiotics, including erythromycin, clarithromycin, and roxithromycin, but not to telithromycin, which is distinct from other macrolides with additional ketone groups and aromatic rings. We hypothesize that the route of entry of macrolides is partially dependent on the Wzy capsular transport system, and that distinct molecular structures of macrolides contribute to differences in bacterial susceptibility. Disc diffusion assays were conducted using Wza knock-outs, Wzc knock-outs, and Wza-Wzb-Wzc triple knock-outs of the *E. coli* K30 strain to test their resistance against erythromycin, clarithromycin, azithromycin, and telithromycin. We observed that a Wza deletion confers greatest resistance to erythromycin and clarithromycin, while the absence of Wzc resulted in mildly increased resistance against these two macrolides. All knock-out strains were observed to have increased but incomplete resistance to telithromycin, and only the Wza single knock-out strain was more resistant to azithromycin. We conclude that Wza is essential for outer membrane penetration of erythromycin and clarithromycin, while telithromycin entry is equally dependent on Wza and Wzc. Azithromycin entry is largely dependent on Wza. However, the antibiotic susceptibilities of the triple knockout results contrasted the Wza single knockout susceptibilities, indicating that there may be other factors involved in susceptibility.

Macrolides are a class of widely-used antibiotics that inhibit bacterial protein synthesis. Their mechanism of action involves binding to the 50s ribosomal subunit and dissociating peptidyl-tRNA from the ribosome to halt polypeptide elongation, resulting in a bacteriostatic effect (1). Most macrolides contain a lactone ring composed of 14 to 16 atoms linked to at least one sugar moiety, as they are derived from the lead optimization of erythromycin (Fig. 1) (2, 3).

Macrolides are effective against infections caused by Gram-positive bacteria, but their hydrophobicity means that Gram-negative bacterial strains are rarely susceptible to macrolides (4). The negatively-charged core region of lipopolysaccharides (LPS) in the outer membrane (OM) of Gram-negative bacteria prevents the passage of large, hydrophobic, and structurally complex molecules, thereby providing intrinsic resistance to nonpolar antibiotics (4). Other resistance mechanisms include antibiotic efflux pumps, enzymatic inactivation, and modification of the ribosomal target site (5). Semi-synthetic derivatives of erythromycin such as ketolides and azalides have structures that improve membrane permeability, acid stability, or ribosomal binding to overcome resistance issues and result in increased activity against certain Gram-negative strains (2).

Capsular polysaccharides are extracellular structures that shield bacteria from the environment and assist in biofilm formation (6). In *Escherichia coli* K30, the assembly and transport of Group 1 capsular polysaccharides are performed by the Wzy-dependent biosynthesis system (Fig. 2), which includes Wza, Wzb, and Wzc (6). Wza is an outer membrane protein that forms a periplasm-spanning capsular polysaccharide transport channel with Wzc. Wzb

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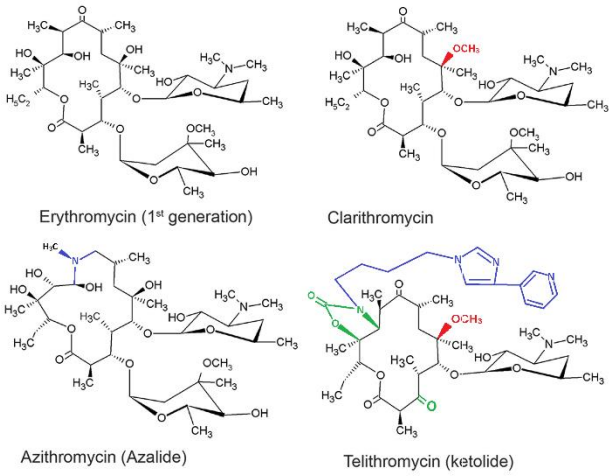


FIG. 1 Chemical structures of macrolide antibiotics used in this experiment with differences from erythromycin highlighted in colour. Black denotes the unchanged erythromycin chemical structure. Red denotes substitution of a hydroxyl group for a methoxy group. Green denotes the addition of a ketone group. Blue denotes the addition of nitrogen containing groups such as a tertiary amine, imine, or imidazole with pyridine. Original picture modified from Gomes *et al.* (13).

is a phosphatase which regulates Wzc activity (6). Wzc is a n inner membrane tyrosine autokinase (6, 7). The structure of Wza consists of eight alpha-helices forming a barrel crossing the OM and is required for capsule polysaccharide export (7, 8). The interior surface of Wza is polar and exposed to the environment, and Wza activity is regulated by the occlusion of the periplasmic region by a flexible loop domain that is manipulated by Wzc (7, 9).

Interestingly, the simultaneous deletion of *wza, wzb*, and *wzc* from the *wzy* cassette in the Gram-negative strain *E. coli* K30 (E69) appears to confer resistance to macrolides (10). Using disc diffusion assays, Botros *et al*. found that a triple deletion of Wza, Wzb, and Wzc in CWG655 conferred increased resistance to erythromycin, clarithromycin, and roxithromycin, but the strain remained sensitive to telithromycin (10). Follow-up projects studying single knockouts have found that both *wza* and *wzc* single deletions conferred erythromycin resistance, while a *wzb* deletion did not (11, 12, 18). The telithromycin sensitivity of CWG655 (Δ*wza-wzb-wzc*) observed by Botros *et al.* shows that the macrolide resistance phenotype does not extend to all antibiotics in this class (10). Erythromycin entry was speculated by Jazdarehee *et al.* to be via the transport channel formed by Wza and Wzc, so that the deletion of the genes encoding either one of those two membrane proteins reduces the permeability of the cell to erythromycin (12).

Based on the model that Wza and Wzc form a channel for macrolide entry, other mechanisms of entry for telithromycin may be utilised to enter Δ*wza-wzb-wzc* cells due to several key differences in the chemical structure of telithromycin that render it comparatively more polar (Fig. 1) (13). Therefore, increased macrolide polarity may result in an improved ability to diffuse across the polar core domain of the outer membrane independently of the Wza-Wzc channel (13).

The prevention of drug entry into a bacterial cell is a significant source of antibiotic resistance (5). Researching the potential mechanism of macrolide resistance due to the absence of the Wzy-dependent biosynthesis system, or necessary constituent proteins may identify molecular trends in antibiotic resistance and susceptibility and identify protein characteristics that permit entry of other commonly available drugs, and avoid excessive stress on new drug design.

Telithromycin is a member of the ketolide subgroup of macrolides that is characterized by several key changes in its chemical structure as compared to erythromycin. First, a sugar and a hydroxyl group on the main lactone ring of erythromycin are respectively replaced with a ketone functional group and a cyclic carbamate group in telithromycin (14). The addition of a large aromatic alkyl-aryl extension group also increases the molecular affinity to the 50s ribosomal subunits (15). These changes increase the molecular polarity and binding strength, resulting in

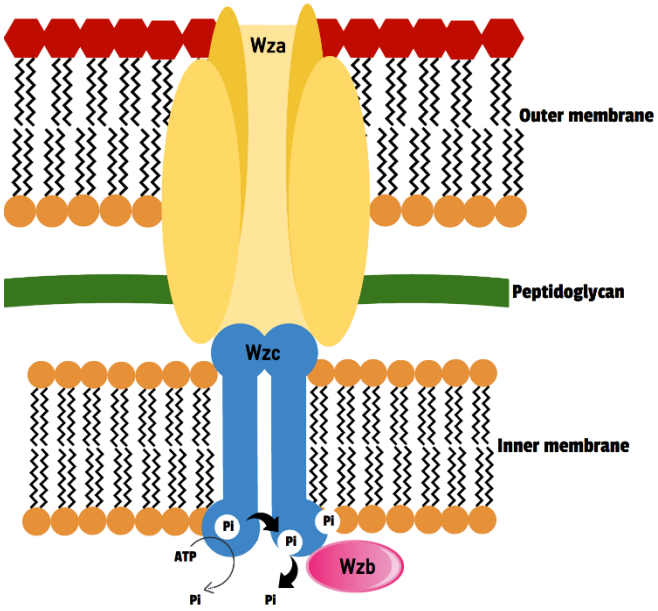


FIG. 2 Diagram of Wza, Wzb and Wzc arranged in a section of the Wzy-dependent biosynthesis system. Capsular polysaccharides are transported across the inner membrane where they interact with Wza, Wzb, and Wzc, and are transferred through the OM (6). Wza forms a channel that spans the OM and transports polysaccharides to the cell surface (6). Wzc is a tyrosine autokinase that is regulated by Wzb, which is a phosphatase (6).

more effective bacteriostatic potency of ketolides when compared to other macrolides (15).

Based on the premise that telithromycin is more effective against Δ*wza-wzb-wzc* strains than erythromycin due to modifications that make the antibiotic more polar, we hypothesized that other macrolide antibiotics that have higher polarity should also be effective against the Δ*wza-wzb-wzc* strain. To investigate this, we performed disc diffusion assays with a range of macrolide antibiotics. Alongside telithromycin, erythromycin and clarithromycin were also tested. Additionally, we included azithromycin in our experiments, which is a macrolide from the azalide subgroup that contains the addition of a nitrogen atom to make a 15-membered lactone ring, resulting in a higher basicity compared to erythromycin (13). The alteration of electrostatic charges allows the molecule to diffuse more readily across the outer membrane than other macrolides (13). Azithromycin is more effective against Gram-negative bacteria compared to erythromycin, while also maintaining a bacteriostatic effect against Gram-positive organisms (3). Azithromycin was not previously tested for susceptibility against *E. coli* capsule assembly knockouts, and is unique from other macrolides in that it is routinely used to treat infections caused by the Gram-negative *Enterobacteriaceae* (13).

We performed several disc diffusion assays using the wild-type (E69), CWG655 (Δ*wza-wzb-wzc*), CWG281 (Δ*wza*), and CWG285 (Δ*wzc*) *E. coli* K30 strains. We included CWG281 and CWG285 strains in our assays because the effects of a single deletion on macrolide resistance have only been studied for erythromycin so far.

MATERIALS AND METHODS

Bacterial Strain Confirmation, Preparation of Media, and Growth Conditions. *E. coli* K30 E69 (serotype: O9a:K30:H12), CWG655 (wza_{22min}::aadA Δ(wza-wzb-wzc)$_{K30}$::aphA3), CWG281 (wza_{22min}::aadA wza_{K30}::aacC1), and CWG285 (wzc_{cps}::aacC1 wza_{22min}::aadA), were obtained from the MICB421 laboratory stock from the Department of Microbiology and Immunology at the University of British Columbia. The strains were originally from the laboratory of Dr. Chris Whitfield, Department of Molecular and Cellular Biology at the University of Guelph. Strain identity was confirmed by plating on antibiotic-supplemented media corresponding to antibiotic resistance cassettes used to create the knockouts, which are kanamycin and spectinomycin for Δ*wza-wzb-wzc* (16), gentamicin and spectinomycin for Δ*wza* (10), and gentamicin and spectinomycin for Δ*wzc* (17). All strains were grown on Luria-Bertani (LB) agar at 37°C supplemented with the following antibiotics where appropriate to identify the strains: kanamycin (100 μg / mL), spectinomycin (100 μg / mL), and gentamicin (10 μg / mL).

Disc Diffusion Assay. The Kirby-Bauer method modified from Su *et al.* was used to measure antibiotic susceptibility (18). Bacterial cells were grown in LB broth (1.0% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl, pH 7) and incubated at 37°C for 18 hours in 5 mL LB on a shaking rack. Prior to spreading, cell concentration was diluted to 1 OD_{660} measured using a Spectronic 20+ spectrophotometer. 100 μL of the 1 OD_{660} culture was spread onto LB plates. Each plate received three antibiotic discs that contained 15 μg of erythromycin, clarithromycin, telithromycin, or azithromycin, which were placed equally spaced apart on one half of the plate using a sterilized forceps. Three blank Whatman paper discs were placed on the other half to serve as the negative control. The plates were incubated for 18 hours at 37°C.

We initially used hand-poured LB agar plates, which had an average volume of 21.4 mL per plate. To control for variation in agar volume, we used an automated dispenser to pour plates that contained exactly 20 mL of LB agar per plate. A total of seven replicates of each antibiotic and strain combination was performed, yielding 21 measurements for each. One plate of Δ*wza-wzb-wzc cells* containing three discs of azithromycin was rejected due to ambiguous zone edges. The zone of inhibition was measured from the edge of the paper disc to the edge of the clear zone using a ruler that had increments of 0.5 mm. Since each person measured the same zones slightly differently, one team member was randomly chosen to measure all zones of inhibition to minimize experimental error.

Statistical analysis. Statistical analysis comparing the zones of inhibition of each bacterial strain treated with a specific macrolide was performed using a paired two tailed t-test ($p<0.05$). Due to an altered population variance from the rejection of three data points of the Δ*wza-wzb-wzc* strain with azithromycin, an additional variance calculation was performed on the strain comparisons to determine if a two-sample equal or unequal variance t-test was appropriate. An equal variance t-test was performed when comparing the Δ*wzc* and Δ*wza-wzb-wzc* strains, and an unequal variance t-test was performed when comparing WT E69 with Δ*wza-wzb-wzc*, and Δ*wza* with Δ*wza-wzb-wzc*.

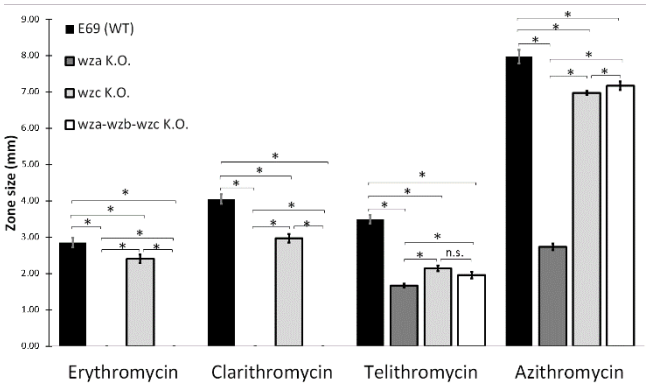
RESULTS

Deletion of Wza confers resistance to erythromycin and clarithromycin. Su *et al.* previously showed that that the deletion of Wza confers resistance to erythromycin (Table 1, Fig. 3) (10, 11, 18). To initiate our study, we first repeated this experiment using strains CWG281 (Δ*wza*), CWG285 (Δ*wzc*), and CWG655 (Δ*wza-wzb-wzc*). Strains of Δ*wza* and Δ*wza-wzb-wzc* both had no observable zone of clearance around erythromycin antibiotic discs, resembling the negative control (Table 1, Fig. 3). The lack of any zone of inhibition indicates that Δ*wza-wzb-wzc* and Δ*wza* strains are resistant to the antibiotic concentration diffused through

TABLE 1 Summary of relative inhibition zone sizes for strains of WT, Δ*wza*, Δ*wzc*, and Δ*wza-wzb-wzc* to erythromycin, clarithromycin, telithromycin and azithromycin via disc diffusion assay on automated dispenser poured plates. Larger zone sizes correspond to more + signs, and no observed zone is represented with a - sign.

	Erythro-mycin	Clarithro-mycin	Telithro-mycin	Azithro-mycin
WT	+	++	++	+++
Δ*wza*	-	-	+	+
Δ*wzc*	+	++	+	+++
Δ*wza-wzb-wzc*	-	-	+	+++

FIG. 3 Average inhibition zones of WT, Δ*wza*, Δ*wzc*, and Δ*wza-wzb-wzc* to erythromycin, clarithromycin, telithromycin and azithromycin via disc diffusion assay on automated dispenser poured plates. Error bars are derived from standard error calculations of the data. * indicates a p-value under or equal to 0.05, and n.s. indicates a non-significant p-value over 0.05.



the agar at the surface. The WT inhibition zone was similar but significantly different to the inhibition zone of Δ*wzc* (fig 3).

We examined clarithromycin resistance due to its similar polarity and structure to erythromycin (Fig. 1), and because Botros *et al.* had also reported that Δ*wza-wzb-wzc* was resistant to clarithromycin (10). There were no observable zones of clearance around clarithromycin antibiotic discs for strains of Δ*wza-wzb-wzc* and Δ*wza* (Table 1, Fig. 3). The WT and Δ*wzc* strains had similar but significantly different susceptibilities to clarithromycin (Table 1, Fig. 3). The lack of Wza in both Δ*wza-wzb-wzc* and Δ*wza* indicates that the removal of Wza has a significant effect in conferring erythromycin and clarithromycin resistance.

Deletion of Wza or Wzc confers a similar phenotype of resistance to telithromycin. Botros *et al.* have previously shown that the effects of telithromycin, a macrolide from the ketolide subgroup, on the Δ*wza-wzb-wzc strain* are similar to the WT strain (10). We repeated this experiment and also included the strains Δ*wza* and Δ*wzc*. We compared the effects of macrolide polarity on susceptibility between telithromycin and the monobasic macrolides erythromycin and clarithromycin. The average inhibition zone sizes for the WT strain to telithromycin were similar to those for erythromycin and clarithromycin (Table 1, Fig. 3). Unlike

with erythromycin and clarithromycin, there were zones of inhibition surrounding telithromycin discs for the Δ*wza*, Δ*wzc*, and Δ*wza-wzb-wzc* strains. The size of each mutant zone was significantly different from the WT (Table 1, Fig. 3). The zone size difference between Δwza was also significantly different from all other strains tested with telithromycin, while the difference between Δwzc and Δwza-wzb-wzc was not significant (Table 1, Fig. 3). We were unable to replicate the observations made by Botros *et al.*, who reported that the WT strain and Δ*wza-wzb-wzc* had comparable susceptibilities to telithromycin (10). From these results, the deletion of Wza or Wzc results in an equal level of resistance to telithromycin.

Single deletion of Wza confers greater resistance to azithromycin than the triple deletion of Wza, Wzb, and Wzc. We examined the effect of genomic deletion of the Wzy system components on azithromycin susceptibility. Azithromycin was chosen because it is from the azalide subgroup of macrolides, has a dibasic structure, and its effectiveness has not been previously studied in *E. coli* capsule assembly knockouts. The use of azithromycin resulted in the largest inhibition zones overall observed in this experiment (Table 1, Fig. 3). The WT, Δ*wza-wzb-wzc*, and Δ*wzc* strains had similar-sized zones of inhibition, and were calculated to be significantly different from each other (Table 1, Fig. 3). Δ*wza cells were* observed to have the lowest susceptibility to azithromycin; however, a notable inhibition zone approximately ⅓ of the WT was still present (Table 1, Fig. 3). From these results, the single deletion of Wza appears to be significantly influential in developing azithromycin resistance, but the additional deletion of Wzb and Wzc results in the resistance being muted.

DISCUSSION

In this study, we examined the susceptibilities of *E. coli* K30 mutants to various macrolide antibiotics. Previous studies using these mutants have found that the deletion of Wza and Wzc confers resistance to erythromycin, while the deletion of Wzb does not (11, 12, 18). However, the effect of other macrolide antibiotics on these knockouts has been largely unexplored.

The thickness of agar used in disc diffusion assays can affect results, because antibiotics on thicker agar tend to diffuse downwards rather than outwards from the disc, resulting in smaller zones of inhibition. This was a factor to consider in our first two trials of the disc diffusion assay, where we used three replicates of hand-poured plates that each contained slight variations in agar volume, leading to variations when measuring zone of inhibition size. We found that the machine-poured plates had more consistent measurements than hand-poured plates, but the trends of susceptibilities across all *E. coli* strains to the macrolides we examined were the same between hand-poured and machine-poured plates.

We initially considered including a minimum inhibitory concentration (MIC) assay in our experiments, however we decided against it, because previous studies by Su et al. and Jazdarehee et al. had

noted that *E. coli* K30 mutants would exhibit erythromycin resistance using the disc diffusion assay but not in the MIC assay (12, 18). These differences were speculated by Jazdarehee et al. to be caused by regulatory gene activation in only submerged colonies, which may interfere with other antibiotic sensitivity assays by incorporating additional unknown gene activities that we could not control for (12). Our initial disc diffusion assays to replicate experiments with erythromycin were consistent with previous studies for strains of Δ*wza-wzb-wzc* and Δ*wza* (Fig. S1) (10, 18). However, we found that the susceptibility of Δ*wzc* cells to erythromycin was similar to the WT strain (Fig. 1, S1), which is in contrast to Jazdarehee *et al.*'s results, who reported that Δ*wzc* strains were more resistant to erythromycin as compared to the WT strain (12). This may have been due to a difference in protocol; we plated 100 µL of bacterial culture in our experiments, while Jazdarehee *et al.* plated 1 mL (12). The extra volume could have diluted the concentration of the antibiotic diffusing from the disc on the agar surface, which would in turn reduce its efficacy against Δ*wzc* and result in smaller zones of inhibition.

The relative susceptibilities of each *E. coli* strain studied to clarithromycin resembled erythromycin, which we hypothesized would occur due to their similar chemical structures and polarities (Fig. 1). Δ*wza* and Δ*wza-wzb-wzc* strains both lack the Wza outer membrane channel and were significantly more resistant to these two antibiotics than the WT strain (Table 1, Fig. 3). The Δ*wzc* strain, which bears a single Wzc deletion, exhibited a similar or slightly lower susceptibility compared to the WT. Wza therefore may be the most important means for erythromycin and clarithromycin to enter the cell, while Wzc has a smaller influence.

We hypothesized that ketone groups which render telithromycin structure more polar than erythromycin (Fig. 1) would allow it to be more effective against knockouts by entering cells without fully relying on the Wza-Wzc entry model speculated by Jazdarehee *et al.* (12). The presence of inhibition zones for Δ*wza* and Δ*wza-wzb-wzc* strains in response to telithromycin (Table 1, Fig. 3) supports the idea that Wza is not the only mode of entry, since erythromycin could not produce a visible zone under the same conditions. A different outer membrane protein, with a polar internal surface like Wza, may also allow telithromycin to enter the periplasm. This could explain why Δ*wza* is less susceptible to telithromycin compared to the WT, but inhibition zones are still present around the discs, unlike with erythromycin (Table 1, Fig. 3). Once in the periplasm, Wzc could assist with transport of telithromycin through the cell membrane. The deletion of Wzc confers a similar increase in resistance to the deletion of Wza (Table 1, Fig. 3), suggesting that this protein may also be important for telithromycin to enter the cell as a sequential interaction from Wza to Wzc. Therefore, disruption of either Wza or Wzc effectively reduces the susceptibility of the cell to telithromycin.

The difference in drug potency between erythromycin, clarithromycin, telithromycin, and azithromycin must also be considered in this analysis. Since we are unable to quantify the uptake of each respective antibiotic, the larger zone size could also be the result of a more potent drug. As a result of the increased binding affinity of telithromycin to the 50S ribosomal subunit the observed zones of clearance for the Δ*wza* and Δ*wza-wzb-wzc* knockouts may also be observed using erythromycin or clarithromycin at a proportionally increased concentration to compensate for potency (15). Future experiments can examine if this trend hold true with these strains at various concentrations of polar and nonpolar macrolide antibiotics. The relatively large inhibition zones sizes of *E. coli* mutants to azithromycin compared to erythromycin (Table 1, Fig. 3) supports our hypothesis that a macrolide with higher polarity is able to enter the cell without completely relying on Wza. The presence of an inhibition zone for Δ*wza* cells suggests that azithromycin may also enter the periplasm through a different outer membrane protein. Alternatively, azithromycin may penetrate the outer membrane through a self-promoted uptake pathway, since the dibasic molecular charge can bind to divalent cationic sites on the LPS, increasing outer membrane permeability (19).

Unexpectedly, the single deletion of Wza in confers significantly more resistance to azithromycin than the triple deletion in Δ*wza-wzb-wzc* cells, even though both of these strains lack Wza (Table 1, Fig. 3). This suggests there may be other cell components that influence azithromycin susceptibility. In *E. coli* K12, the RcsC sensor kinase forms a two-component system with a response regulator RcsB, which regulates a variety of genes that allow the bacterial surface to adapt to its environment (20). RcsC can respond to several different signals, one of which being changes in levels of cell surface proteins (20). It is possible that the Wza-Wzb-Wzc triple deletion mutant decreased the amount of cell surface protein below a threshold level, triggering a RcsC response that leads to an upregulation of other

membrane proteins. The additional membrane proteins may provide an alternative pathway for azithromycin entry. The single deletion of Wza may not lower the surface protein enough to trigger an upregulation response, so without an alternative route less azithromycin enters the cell, rendering it more resistant than the triple deletion. It is also possible that the phosphatase Wzb plays a role, since Δ*wza* cells have a functioning *wzb* gene while Δ*wza-wzb-wzc* cells do not. To further investigate this hypothesis, we attempted to test the resistance of Δ*wzb* cells, but the strain showed very poor growth and cannot be used for disc diffusion assays.

Alternative explanations for the mutant strains resistance to macrolides are possible. The ineffective distribution of capsular polysaccharides in mutants may induce the expression of an antibiotic efflux transporter. An efflux transporter would require that macrolide penetration through the OM is independent of the Wzy system, and instead rely on a slow rate of diffusion across the lipid bilayer (21). The large size of macrolides would make the efficient export of the drug unlikely (21). The loss of Wza, or to a lesser extent Wzc, may be sufficient to induce the expression of an efflux protein that efficiently transports erythromycin and clarithromycin out of the cell to result in the observed phenotype (Table 1, Fig. 3). The efflux protein may have a reduced affinity to telithromycin, resulting in the broadly similar zone sizes observed for all mutant strains. However, this explanation does not explain the peculiar differences in susceptibility between the Δ*wza* and Δ*wza-wzb-wzc* strains when challenges with azithromycin (Table 1, Fig. 3). Therefore, the involvement of an efflux transporter is unlikely.

Based on the macrolide antibiotic disc diffusion assays we conclude that Wza is a possible prerequisite for susceptibility to erythromycin and clarithromycin, while Wzc has a similar, but smaller influence. It is likely that the uptake of erythromycin and clarithromycin are largely dependent on Wza. The entrance of telithromycin, however, may be equally dependent on Wza and Wzc, as the knockout of either and both of these proteins resulted in similar levels of resistance to telithromycin, but failed to induce full resistance. The results potentially support our hypothesis that the polarity of macrolides will affect the route of entry, thereby altering susceptibility, however more polar and nonpolar antibiotics need to be tested at different concentrations to further scrutinize the mechanism of this potential drug-protein interaction. Telithromycin is a more polar molecule possessing extra ketone groups, methoxy groups, and aromatic rings, all of which increases its binding ability (Fig. 1). It is likely that the entry of telithromycin is partially dependent on the Wzy system as a whole, yet its unique properties allows it to enter the cell from another unrelated pathway, maintaining a baseline level of susceptibility. Azithromycin, which also has higher polarity (Fig. 1), may be able to enter the cell without Wza. However, since the single Wza deletion is more resistant than the triple deletion, this suggests there are other factors that influence azithromycin susceptibility in *E. coli* K30.

FUTURE DIRECTIONS

The loss of Wza in CWG281 (Δ*wza*) confers an increased resistance to azithromycin as compared to the loss of Wza, Wzb, and Wzc in CWG655, though we had expected that a lack of Wza in both of these strains would confer similar levels of resistance. A future study investigating the reason behind this may find cell components other than Wza and Wzc that influence azithromycin susceptibility in *E. coli* K30. The role of Wzb in azithromycin resistance of CWG281 (Δ*wza*) may be easiest to investigate first. Though Rana *et al.* have determined that the deletion of Wzb is not involved in erythromycin resistance, it may be interesting to see if this also holds true for azithromycin (11). One might perform disc diffusion assays for azithromycin comparing CWG655 (Δ*wza-wzb-wzc*), CWG281 (Δ*wza*), CWG343 (Δ*wzb*), and CWG344 (Δ*wza-wzb*).

Future experiments could examine other macrolides, such as roxithromycin, which we were unable to obtain. Additional macrolides that have higher polarity may be approved in the future, such as the fluoroketolide solithromycin (22). The proposed self-promoted uptake pathway of azithromycin could also be investigated by varying the concentration of dissolved magnesium ions in the agar to compete with azithromycin in binding to the LPS layer (19).

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