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Deletion of *Escherichia Coli* K30 Type I Capsule Assembly Gene *wzc* Confers Resistance to The Antibiotic Erythromycin in Solid Media

Aria Jazdarehee, Jennifer Joy Anderson, Devon Morrison, William Pardoe
Department of Microbiology & Immunology, University of British Columbia

***Escherichia coli* K30 produce a Type I (Colanic Acid) capsule which protects the bacterium from environmental stresses, including host immune responses. It is unknown how the presence of Type I capsule affects antibiotic resistance in the bacterium. Previous studies have shown that the deletion of three key genes involved in Type I capsule production, *wza*, *wzb*, and *wzc*, confers increased resistance to erythromycin on solid media. It has been found that the single deletion of *wza*, but not *wzb*, is sufficient to confer this resistance phenotype. In this study, we investigated the effects of deleting the *wzc* gene on erythromycin sensitivity in *E. coli* K30. We tested erythromycin sensitivity in wild-type (WT) *E. coli* K30 strain E69 to knockout mutants lacking either the *wza* or *wzc* genes. On solid media, we observed that the deletion of *wza* and *wzc* confers increased resistance to erythromycin compared to WT cells. In contrast, assays completed in liquid media produced inconsistent and inconclusive results. Our results support the hypothesis that the Wza and Wzc proteins are involved in translocating macrolide antibiotics, such as erythromycin, into the cell.**

Over the past two decades, the number of infections caused by antibiotic-resistant bacteria has increased, sparking an interest in the mechanisms involved in conferring resistance to these organisms (1). In addition to intrinsic barriers and structures, bacterial resistance to antibiotic has been shown to be achieved through numerous mechanisms, thus complicating treatment strategies (1,2). An understanding of the mechanisms involved in conferring resistance to antibiotics, as well as what mechanisms cause sensitivity, is required in order to combat this threat (3).

Macrolides are a class of antibiotics which inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit of prokaryotic ribosomes (4). Gram-negative bacteria, including *Escherichia coli*, are intrinsically resistant to macrolides such as erythromycin as they cannot cross the outer membrane lipid bilayer barrier (5,6). However, several studies have shown that the *Escherichia coli* K30 E69 strain is susceptible to erythromycin treatment (7,8,9). One relevant characteristic of the susceptible *E. coli* K30 strain is the production of the Type 1 (K30) capsule, which functions to protect the bacterium from environmental stresses, such as the host immune system (10). The capsule consists of repeating polysaccharide units resembling colanic acid (10). Though it has been postulated that this capsule increases the invasiveness of the bacterium, it is still unknown how the presence of the Type 1 capsule impacts antibiotic resistance in *E. coli* K30 (10).

Biosynthesis of the Type 1 capsule requires the highly conserved *wzy* gene cassette, which consists of four genes: *wza*, *wzb*, *wzc*, and *wzi* (10). The *wza*, *wzb*, and *wzc* genes encode for proteins which are essential for the biosynthesis of the capsule (10) (Fig. 1). Wza is an outer-membrane lipoprotein which forms a capsule translocation channel (11). Wzc is a large inner-membrane autokinase with multiple domains, one of which forms part of a capsule translocation pore spanning the inner membrane and periplasm (10). The activity of Wzc is regulated by a C-terminus autokinase domain and by the phosphatase activity of Wzb (10). While the production of capsule has thought to play a protective role, Botros *et al.* recently showed that the deletion of the *wzy* gene cassette confers increased resistance to erythromycin compared to WT *E. coli* K30 cells (7).

Su *et al.* then reported that the single deletion of the *wza* gene was sufficient to confer the resistant phenotype observed in the *wzy* gene cassette knockouts compared to WT cells (8). They proposed that Wza may play a role in translocating erythromycin across the outer membrane, noting that the Wza pore size is large enough to accommodate the antibiotic (8). This notion was further supported by Rana *et al*., who later found that the deletion of the *wzb* gene alone is not sufficient to confer this resistant phenotype (9). While these studies suggest Wza may play a role in translocating erythromycin into the bacterial cytosol, further study is needed to investigate the mechanism in

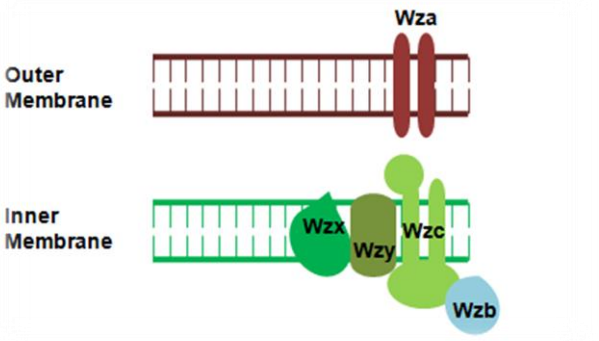

FIG. 1 Simplified model of *wzy*-dependent production of group I capsule in *Escherichia coli* K30. Wza is an outer membrane channel which associates with Wzc, an inner membrane protein which forms part of the capsule translocation pore in conjunction with inner membrane proteins Wzx and Wzy. Wzb is a cytosolic phosphatase which associates with Wzc.

which the deletion of the *wzy* gene cassette confers resistance to the antibiotic. Studies have not yet investigated the role Wzc plays in conferring this resistant phenotype.

Building upon the notion set by Su *et al.*, we sought to investigate the role Wzc plays in conferring resistance to erythromycin. To achieve this, we studied whether the deletion of the *wzc* gene confers increased erythromycin resistance compared to WT *E. coli* K30. We hypothesized that the deletion of *wzc* would result in increased resistance to erythromycin compared to WT cells. We predict that the antibiotic enters the cell by translocating through the pores formed in the inner and outer membranes, by Wza and Wzc, respectively. It has been shown that a large periplasmic domain of Wzc interacts closely with Wza to facilitate capsule export out of the cell, so this close association may also facilitate entry of erythromycin into the cell (12).

Here we report that the deletion of the *wzc* gene in *E. coli* K30 confers increased resistance to erythromycin compared to WT *E. coli* K30 in assays completed on solid media but not assays completed in liquid media. Our work supports the notion that Wzc plays a role in translocating erythromycin into the cell, but also brings to light important discrepancies in *E. coli* K30 phenotypes in solid media versus liquid media.

MATERIALS AND METHODS

Bacterial strains, media, and culture growth conditions. *E. coli* K30 CWG343 (E69 WT), CWG285 (Δ*wzc*), and CWG281 (Δ*wza*) strains were obtained from the laboratory of Dr. Chris Whitfield from the Department of Molecular and Cellular Biology, at the University of Guelph. In each experiment, liquid cultures of bacteria were grown at 37^{O}C on a shaker at 200 rpm, in a walk in incubator. Cells cultures were grown in Luria Bertani (LB) broth. The liquid broth had a chemical composition of 0.5% w/v NaCl, 1.0% w/v tryptone, and 0.5% w/v yeast extract, at a pH of 7.0. The solid LB plates had the same composition supplemented with 1.5% agar as a solidifying agent.

Broth dilution minimum inhibitory concentration (MIC) assay. The MIC assay was used to determine the relative minimum inhibitory concentrations of the *E. coli* K30 *wzc* and *wza* knockouts in comparison to the WT strain. A protocol modified from Rana *et al.* was used (9). Overnight cultures were prepared by inoculating 5 ml aliquots of LB with each strain and grown at 37^{O}C and 200rpm for 24 hours. Each culture was measured using a Spectronic 20+ spectrophotometer and diluted with LB to an OD660 of 0.0001. A 10mg/mL stock solution of erythromycin was made by adding 0.05g of erythromycin rehydrate to 5ml of 100% ethanol. The stock solution of erythromycin was then diluted with 100% ethanol to 2000μg/ml prior to use in each MIC assay. A 96-well plate was set up for each strain used in the MIC assay and 100μL of LB was pipetted into each well. Next, 200μL of the diluted erythromycin was added to each well in the first column, and 100μL was transferred from column 1 to column 2 to using a multichannel pipette which was mixed by pipetting up and down. Serial dilutions were continued for columns 1 through 11 resulting in a two-fold decrease in antibiotic concentration in adjacent columns. Resulting antibiotic concentrations are listed in Table 1. Column 12 acted as a positive control as no antibiotic was added. A 10μL aliquot of the corresponding overnight culture was added to each well of columns 1 through 10 and column 12. Column 11 acted as a negative control with no cells added. Plates were incubated for 20 hours at 37^{O}C in a walk-in incubator. A qualitative analysis of the plate was done by visually characterizing each well as having growth or no growth. Wells were classified as having heavy growth if the culture was opaque, and light growth if the culture was translucent.

Disc diffusion assay. A protocol modified from Rana *et al.* was used (9). Overnight cultures were prepared by inoculating 5 ml aliquots of LB with each strain and grown at 37^{O}C and 200rpm for 24 hours. Each culture was measured using Spectronic 20+ spectrophotometer and diluted with LB to an OD660 of 1.5. A volume of 1mLof each culture was plated on an LB agar plate. Three erythromycin antibiotic discs were equally spread on half of the LB plate and three Whatman paper discs of the same size were similarly spread on the other half, acting as a negative control. Each plate was set up in duplicate to ensure accuracy of the results. All six plates were placed in the walk-in incubator for 20 hours at 37^{O}C. A qualitative analysis of the plate was done by characterizing the presence or absence of a zone of inhibition around each disc. Zones were defined by the visual characterization of an area of no cell growth surrounding the disc.

RESULTS

Deletion of the *wza* or *wzc* Gene is sufficient to confer resistance to erythromycin in solid media. The Δ*wza* strain (CWG281)**,** which is an *E. coli* K30 strain containing a knockout of the *wza* gene in the *wzy* gene cassette, was used to confirm the previous finding that the deletion of the *wza* gene is sufficient to confer erythromycin resistance in *E. coli* K30 (8,9). Using a modified version of the disc diffusion assay described by Rana *et al.*, we tested the susceptibility of the *wza* and *wzc* single knockouts to erythromycin in comparison to the WT *E. coli* K30 strain (9). The WT cells had large zones of inhibition around all Erythromycin discs. In comparison, the erythromycin discs on the Δ*wzc* plates had very small zones of inhibition, approximately ¼ of the size of the zones of inhibition seen on the WT plate. Four of the six erythromycin discs on the Δ*wza* plates had zones of inhibition approximately ½ the size of the zones of inhibition on the Δ*wzc* plate, and the remaining two of the erythromycin discs had no zones of inhibition. None of the Whatman paper discs on any of the plates had a zone of inhibition (Data not depicted).

Erythromycin MICs in *E. coli* K30 are inconsistent and inconclusive in liquid media. A broth dilution MIC assay was then performed to confirm the results observed

TABLE 1 Erythromycin concentrations used in the MIC assay. Table 1 shows is the setup of the 96-well plate in the MIC assay. The number in each well represents the concentration of erythromycin present. All concentrations are given in μg/mL. Column 11 acts as a negative control with no cells added. Column 12 acts as a positive control with no antibiotic added

	1	2	3	4	5	6	7	8	9	10	11	12
A	1333	667	333	167	83	42	21	10	5	2.6	(-)	(+)
B	1333	667	333	167	83	42	21	10	5	2.6	(-)	(+)
C	1333	667	333	167	83	42	21	10	5	2.6	(-)	(+)
D	1333	667	333	167	83	42	21	10	5	2.6	(-)	(+)
E	1333	667	333	167	83	42	21	10	5	2.6	(-)	(+)
F	1333	667	333	167	83	42	21	10	5	2.6	(-)	(+)
G	1333	667	333	167	83	42	21	10	5	2.6	(-)	(+)
H	1333	667	333	167	83	42	21	10	5	2.6	(-)	(+)

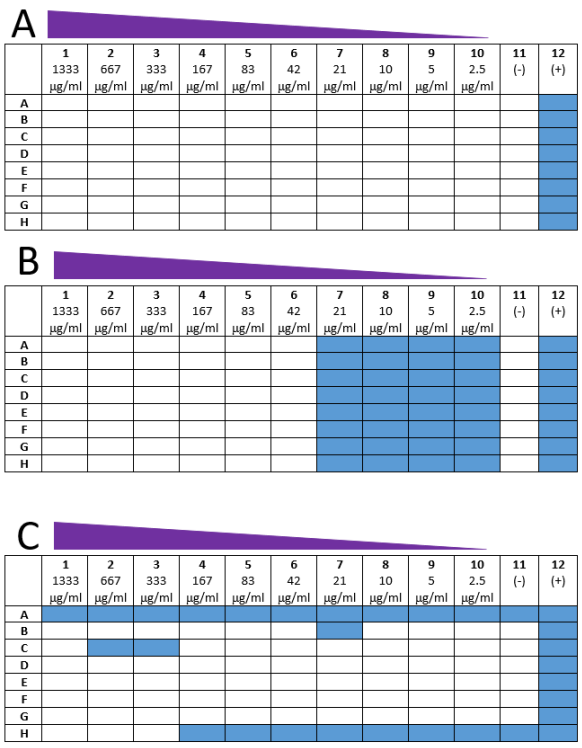

FIG. 2 Erythromycin MIC is 42µg/ml for the *wzc* knockout strain. Figure 2 demonstrates the growth in each well in the 96-well plate for the MIC assay. Dark blue represent opaque cultures and light blue represents transparent cultures. Concentration of erythromycin increases from column 10 to column 1. "A" represents the susceptibility of the WT strain to erythromycin. "B" represents the susceptibility of the Δ*wza* strain to erythromycin. "C" represents the susceptibility of the Δ*wzc* strain to erythromycin.

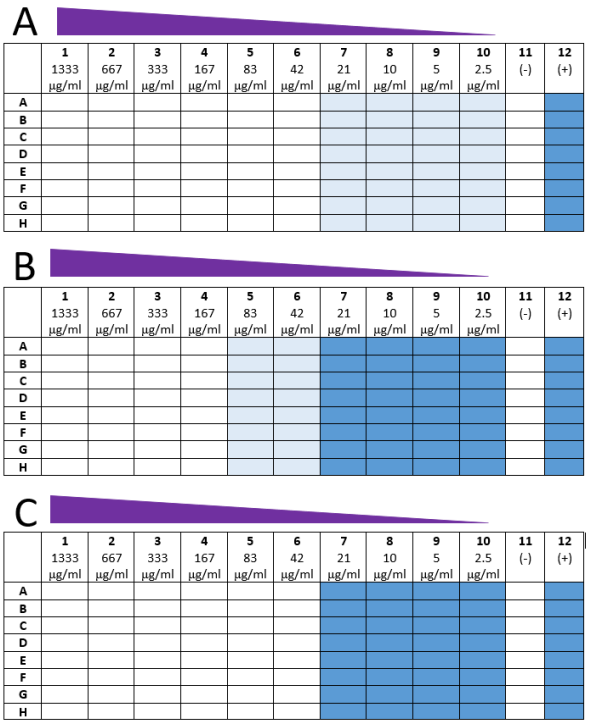

FIG. 3 *wzc* knockouts display increased resistance to erythromycin compared to WT *E. coli* K30. Figure 3 demonstrates the growth in each well in the 96-well plate for the MIC assay. Dark blue represent opaque cultures and light blue represents transparent cultures. Concentration of erythromycin increases from column 10 to column 1."A" represents the susceptibility of the WT strain to erythromycin. "B" represents the susceptibility of the Δ*wza* strain to erythromycin. "C" represents the susceptibility of the Δ*wzc* strain to erythromycin.

with the disc diffusion assay. Three trials of the MIC assay were performed, with each trial demonstrating a different results. A protocol modified from Rana *et al.* was used (9). A 96-well plate was set up for each strain with two-fold decreases in antibiotic concentration in each column. The concentrations of each column of the 96-well plate are depicted in Table 1.

In the initial trial, there was no growth of the WT strain in columns 1 through 11, but growth was observed in the positive control lane (column 12) (Fig. 2A). In comparison, the observed MIC for the *wzc* knockout strain was 42µg/ml (Fig. 2B). The *wza* knockout strain plate demonstrated bacterial growth around the wells at the edges of the plate, including the negative control, and not in the wells at the center (Fig. 2C).

In the second trial the observed MIC for the WT cultures was 42µg/ml (Fig. 3A). The *wzc* knockout cultures demonstrated heavy growth until an antibiotic concentration of 42µg/ml, but light growth was observed until an antibiotic concentration of 167µg/ml (Fig. 3B). The *wza* knockout cultures demonstrated heavy growth until an antibiotic concentration of 42µg/ml (Fig. 3C).

The third trial demonstrated inconsistent results with the previous two trials. All three plates demonstrated light growth with cultures growing to high concentrations of the antibiotic along the top rows of the plate but only low concentrations along the bottom rows of the plate (Fig. 4).

DISCUSSION

Reverse paradigm. Bacterial capsule is typically thought to be advantageous to capsule-producing strains, conferring resistance to antibiotics and enhanced virulence (10,13). While this paradigm often holds true, we hypothesize that, in the case of erythromycin resistance in *E. coli* K12, the proteins responsible for capsule translocation result in the bacterium being sensitive to erythromycin, not resistant to it, thus reversing the paradigm. However, the presence of capsule may confer advantages to the cell, which was not evaluated in the present study.

Location of erythromycin in the cell. Erythromycin is a bacteriostatic antibiotic that has been shown to bind and inhibit the function of the 50S prokaryotic ribosomal subunit (4), thus inhibiting protein synthesis. As such, it must enter the bacterial cytosol in order to inhibit cell

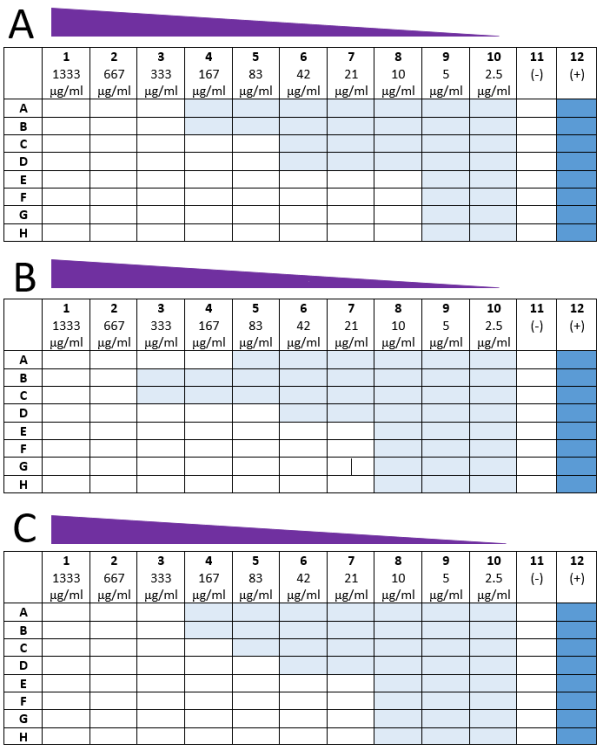

FIG. 4 Results of the MIC assay trial 3. Figure 4 demonstrates the growth in each well in the 96-well plate for the MIC assay. Dark blue represent opaque cultures and light blue represents transparent cultures. Concentration of erythromycin increases from column 10 to column 1."A" represents the susceptibility of the WT strain to erythromycin. "B" represents the susceptibility of the Δwza strain to erythromycin. "C" represents the susceptibility of the Δwzc strain to erythromycin.

growth. Specifically, we hypothesize that the antibiotic enters the cell by translocating through the pores formed in the outer and inner membranes, by Wza and Wzc, respectively. The results of our disc diffusion assay support our hypothesis, phenotypically, that both Wza and Wzc are necessary for the translocation of erythromycin into the cell.

Su *et al.* speculate that erythromycin would fit through the smallest pore created by Wza (8). As it would be necessary for capsule monomers to pass through both the inner and outer membrane pores, we logically hypothesize that erythromycin would also be able to pass through the pore formed by Wzc.

Incongruity of MIC aAssays and disc diffusion assays. While the disc diffusion assay supports our hypothesis, the results of the MIC assays were not conclusive. Su *et al.* found that *wza* knockouts were more susceptible to erythromycin than WT cells and Rana *et al.* found that the *wza* knockouts and WT cells had a similar resistance phenotype (8,9). Our results in the disc diffusion assays were comparable to previous studies, however, our results in liquid media were inconsistent and inconclusive. We suggest that the aforementioned incongruity is due to the underlying differences between the growth conditions of the assays; a disc diffusion assay is performed on a solid medium (an agar plate), while an MIC assay is completed in a broth medium (a 96-well polypropylene plate).

The Regulator of capsule synthesis (Rcs) two-component system has been shown to regulate capsule biosynthesis (14). Specifically, RcsC, a membrane protein, interacts with DjlA and DnaK to respond to hyperosmotic stress (14). Sensor proteins may be acting differently in different media types, leading to different phosphorylation states of RscC, and thus different expression of the capsule biosynthesis genes. It is possible that in liquid culture, due to its relatively stable osmolarity, the expression of capsule biosynthesis genes is down-regulated, leading to the observed lack of difference between the WT and *Δwza* or *Δwzc* mutants.

Conclusions. For the *wzy* gene cassette of *E. coli* K30 E69, many studies have examined the erythromycin resistance of the WT strain in comparison to subsequent knockout strains. Botros *et al.* began by knocking out the essential genes, *wza*, *wzb*, and *wzc*, from the *wzy* gene cassette (7). They found that the deletion of the *wzy* gene cassette confers an increase in macrolide resistance (7). With the focus on erythromycin transport through the *wzy* gene cassette, Su *et al.* and Rana *et al.* knocked out Δ*wza* and Δ*wzb*, respectively (8,9). The results from the studies support that the deletion of *wza* is sufficient to confer antibiotic resistance, while the deletion of *wzb* is not. From there, our study elucidated that *wzc* deletions are also sufficient to provide *E. coli* K30 with resistance to erythromycin. Our results provide support for the hypothesis that Wza and Wzc are involved in the translocation of erythromycin into the cell. Ultimately, a broader understanding of the mechanisms involved in antibiotic resistance and susceptibility allows for the development of novel strategies to combat antibiotic resistant bacteria.

FUTURE DIRECTIONS

There are noticeable inconsistencies between the results obtained from the MIC assays and the disc diffusion assays. Growth of the bacteria in liquid media appeared to yield variable culture densities over three trials, and thus did not serve as a reliable control to test antibiotic resistance. The WT and knockout strains all exhibited trials of complete, partial and no resistance at similar concentrations, while the disc diffusion assays supported that knocking out the *wza* and *wzc* genes consistently increased resistance. In addition to our project, both Su *et al.* and Rana *et al.* similarly found that the two assays reveal contrasting results when tested against the same knockout strain—particularly the Δ*wza* (8,9). Capsule growth in liquid versus solid medium is a proposed explanation, but it would be beneficial to further examine the differences that are being reproduced. The experiments performed will be much more reliable when

we have a more reliable hypothesis to explain each phenotype.

Further study concerning the localization of the antibiotic should be undertaken in order to support this hypothesis mechanistically. We hypothesize that in a Δ*wza* strain, the antibiotic would localize outside of the cell, and that in a Δ*wzc* mutant, the antibiotic would localize to the periplasm. In both cases, the absence of a functional translocation pore prevents the antibiotic from reaching its target in the cytosol. Preliminary studies by Finete *et al.* have described a fluorimetric method for the localization of erythromycin in the cell (16). This experiment would provide some valuable information regarding how macrolides use the proteins to enter the cell and potentially suggest a mechanism that each individual gene contributes to the *wzy* gene cassette. One could advance this direction and test whether other macrolides act similarly to erythromycin, given their similar structures, and then expand this idea using different types of antibiotics.

Finally, we suggest that antibiotic susceptibility tests be performed on a Δ*wzb*c double-knockout to further support the importance of *wzc* with respect to erythromycin resistance.

We attempted to perform a Lambda-Red Recombinase protocol to create a Δ*wzac* knockout, but were unsuccessful in completing the recombination. We produced stocks of chloramphenicol (pKD3) inserts flanked with *wza* and *wzc* genes, respectively. We also transformed pKD46 (containing lambda-red genes) into competent Δ*wzc* cells. The stock solutions will be available for future experiments to save time during the creation of the Δ*wzac*. Primer dimers and non-specific products were continually produced during the generation of pKD3 inserts . Using the pKD3 inserts, it would be best to purify the correct bands from a gel and clone them to obtain sufficient concentrations of DNA. Preliminary data for the generation of pKD3 inserts is depicted in the Supplementary Results section.

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