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Plasmid-mediated complementation of *wza* restores erythromycin susceptibility in *Escherichia coli* K30 strain CWG281

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SUMMARY Group 1 capsules are recognized as virulence determinants in bacterial pathogens of humans and animals. They enhance virulence by conferring resistance to phagocytosis and increasing adherence to host tissues. The assembly and transport of type 1 capsular polysaccharide in *Escherichia coli* K30 is mediated through the Wzy-dependent pathway. This pathway involves: Wza, an outer membrane channel; Wzb, a cytosolic phosphatase; and Wzc, an autokinase in the inner membrane. Previous research has shown that deletion of Wza confers resistance to erythromycin. It has been suggested that the route of entry of erythromycin is partially dependent on the Wza channel. In this study, we hypothesize that plasmid-mediated complementation of *wza* will restore erythromycin sensitivity in an *E. coli wza* knockout mutant. The *wza* gene was previously cloned into an arabinose-inducible pBAD24 vector and transformed into the *wza* knockout mutant. Using a modified arabinose-antibiotic disc diffusion assay, 1% arabinose was observed to induce optimal pBAD24-*wza* expression without impairing cell growth and appropriately evaluate erythromycin sensitivity in the *wza* knockout mutant. We found that, when induced, pBAD24-*wza* transformants showed higher erythromycin sensitivity compared to the control strains without the plasmid. Our findings indicate that plasmid-mediated complementation of *wza* gene restores erythromycin sensitivity in the *wza* knockout mutant strain.

INTRODUCTION

In *Escherichia coli*, capsular polysaccharides (CPS), or K-antigens, serve as a major protection barrier from environmental stresses such as the host immune system (1). In particular, group 1 capsules are recognized as virulence determinants in bacterial pathogens of humans and animals (1). They enhance virulence by conferring resistance to phagocytosis and increasing adherence to host tissues (1).

The role of CPS in antibiotic resistance of *E. coli* is not well understood (2). Previous studies have reported capsule-dependent resistance to kanamycin in *E. coli* (3,4). Moreover, it has been suggested that capsules do not confer resistance to tetracycline or streptomycin (5). Therefore, the capsule-dependent antibiotic resistance may be dependent on the class of antibiotic. *E. coli* K30 strain E69 has been observed to be susceptible to erythromycin treatment, despite the synthesis of group 1 CPS (2).

Macrolides, such as erythromycin, are a class of hydrophobic and non-polar antibiotics containing macrocyclic lactone rings (2) .They bind to 50S ribosomal subunit to inhibit protein synthesis (2). In Gram-negative bacteria, the presence of negatively charged core region of lipopolysaccharides (LPS) in the outer membrane (OM) may confer intrinsic resistance to macrolides by decreasing membrane permeability (6, 7). Divalent cations in the environment are proposed to cross-bridge neighboring anionic LPS molecules, leading to strong lateral interactions between LPS (8). This results in low fluidity and low permeability of OM to large hydrophobic molecules such as macrolides (8). Due to this property, infections

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with Gram-negative bacteria are not usually treated with macrolides (8).The Gram-negative OM thus confers intrinsic resistance to macrolides in most *E. coli* strains (8).

The assembly and transport of group 1 CPS is through the Wzy-dependent biosynthesis pathway (1), which consists of three key proteins: Wza, the outer membrane transport channel; Wzc, the inner membrane autokinase associated with Wza; and Wzb, the cytosolic phosphatase regulating Wzc activity (Fig. 1A) (6). Wzc interacts with Wza in the outer membrane to transport CPS polymers onto the cell surface from the periplasm (7). While capsule production has thought to play a protective role, Botros *et al.* demonstrated that deletion of *wza-wzb-wzc* cluster resulted in increased resistance to erythromycin relative to the wild-type (WT) *E. coli* K30 E69 cells (9). Further, Su *et al*. and Jazdarehee *et al*. have reported, respectively, that single deletion of *wza* or *wzc* in *E. coli* K30 E69 confer erythromycin resistance on solid media (2,10). Conversely, Rana *et al.* found that single deletion of *wzb* is not sufficient to confer resistance to erythromycin (11). Thus, it has been suggested that group 1 CPS production and the Wzy-dependent biosynthesis pathway, specifically Wza and/or Wzc, may play a role in erythromycin transport across the OM and into the cytoplasm of *E. coli* K30 (2,10).

Wza has been proposed to act as a pore through which erythromycin can pass into the cytoplasm (Fig. 1B) (7). Wza transport channel has a diameter of 17 Å, exceeding the estimated size of erythromycin (10.67 Å by 11.97 Å) and can therefore accommodate the antibiotic entry into the cell (2). However, the residues enclosing the alpha-helical Wza channel are mostly hydrophilic, which may hinder the movement of the hydrophobic erythromycin across the channel (2). Based on this information, the role of Wza in erythromycin transport needs to be further explored.

In order to investigate the role of Wza in erythromycin sensitivity, Pochanart *et al*. cloned *wza* into an arabinose-inducible pBAD24 vector (7). The vector allows tight control of *wza* expression in cells through addition of carbon sources to media: L-arabinose acts as an inducer while L-glucose acts as a repressor (7). The tight regulation of protein expression was thought to be suitable for the experiment as Yuen *et al.* observed overexpression of *wza* is associated with lethal phenotype (12). They suggested that reduced cell viability may be due

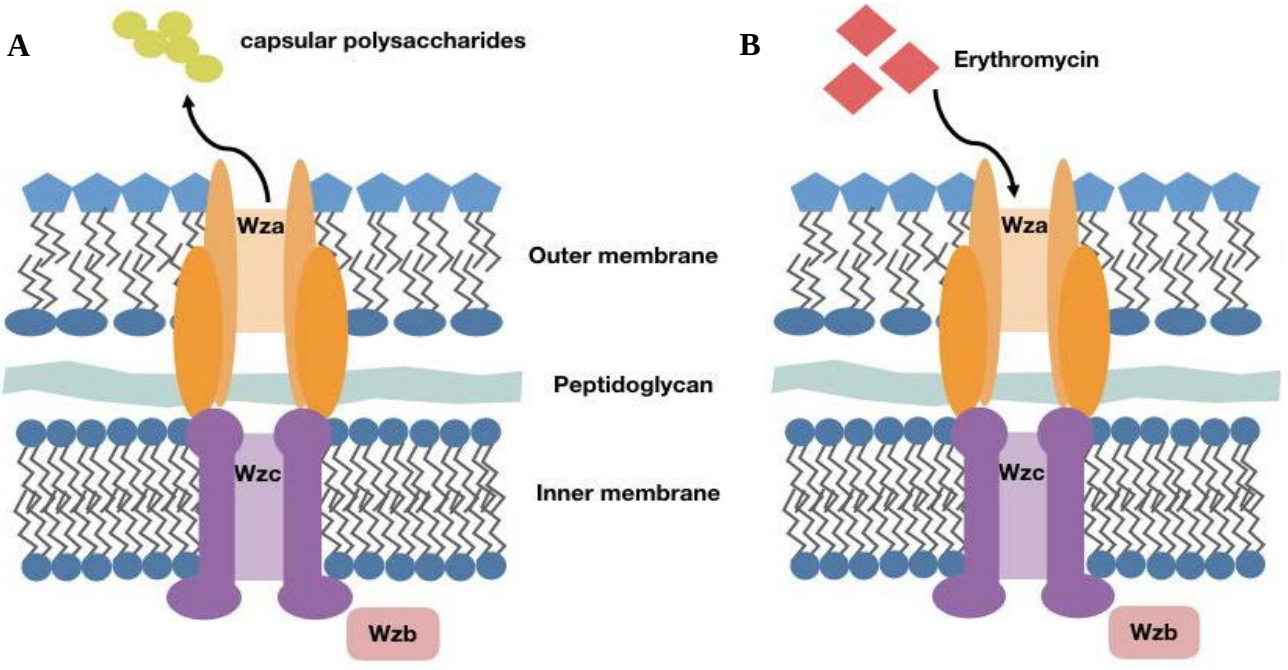

FIG. 1 Simplified model of the Wzy system. (A) Wza and Wzc interact to transport the capsular polysaccharides to the cell surface. Wzb is a cytosolic phosphatase that associates with Wzc. (B) Proposed model for the entry of erythromycin in *E. coli* K30. Erythromycin passes through the Wza outer membrane channel. It is subsequently transported into the cytosol through the interaction of Wza and Wzc.

to the insertion of excess OM pores leading to the destabilization of the OM (12). Damage and loss of the OM results in the increased passage of large molecules through the membrane that leads to bacterial toxicity (13). Thus, the optimal arabinose concentration for pBAD24-*wza* expression must be determined such that it restores the erythromycin-sensitive phenotype without impairing the growth of bacteria. In attempt to optimize arabinose induction, Pochanart *et al*. constructed growth curve analysis in liquid media while assessing erythromycin sensitivity on solid media (7). They found that the sensitivity is restored only at specific L-arabinose concentrations, with 0.008% being the optimal concentration (7).

In this study, using the pBAD24-*wza* constructed by Pochanart *et al*., we developed an arabinose disc diffusion assay on solid medium to find the optimal concentration of L-arabinose needed to restore erythromycin sensitivity in *E. coli* CWG281, *wza* knockout mutant. Since erythromycin sensitivity and bacterial viability was found to be dependent on the level of L-arabinose induction (7), optimization of L-arabinose was necessary (7). Distinct growth curves can result in liquid versus solid media (14). This might be due to the difference in the surrounding environment for microorganisms (14). Therefore, we decided to conduct the optimization of L-arabinose concentration and test the erythromycin sensitivity on the same type of medium.

We hypothesized that plasmid-mediated complementation of *wza* in CWG281 restores erythromycin sensitivity at a specific concentration of L-arabinose on solid media. To test this, we performed a modified arabinose-antibiotic disc diffusion assay to assess the erythromycin sensitivity of CWG281 containing pBAD24-*wza*. In this study, it was found that the Complementation of *wza* in *E. coli* CWG281 restored erythromycin sensitivity at 1% arabinose induction on solid media.

METHODS AND MATERIALS

Bacterial strains, plasmids and growth conditions. The WT *E. coli* K30 strain E69 (serotype: O9a:K30:H12) and the *wza*-knockout mutant CWG281(*wza* $_{22\text{min}}$ ::*aadAwza* $_{K30}$ ::*aacC1*) were obtained from the laboratory of Dr. Chris Whitfield at the University of Guelph (1). The *E. coli* E69 isolate contains two copies of the *wza-wzb-wzc* cluster (1). One copy resides in the *cps* locus and the other copy is situated at min 22 on the *E. coli* K-12 linkage map and is found in many *E. coli* isolates (1). CWG281 is an E69 derivative with a polar spectinomycin-resistance cassette inserted in the *wza* region that eliminates expression of the 22-min copy of the *wza-wzb-wzc* cluster (1). The second copy of *wza* in the *cps* locus was knocked out through the insertion of a gentamicin resistance cassette (1). Previously constructed by Pochanart *et al.*, CWG281 transformants containing either pBAD24-*wza* or pBAD24-*GFP* were also used in this study (7). All growth experiments were performed at 37°C. Bacterial strains were streaked on Luria Bertani (LB) agar plates (0.5% w/v NaCl, 1.0% w/v tryptone, 0.5% w/v yeast extract and 1.5% agar). Isolated colonies were selected to prepare overnight (LB) cultures incubated on a shaker at 200 rpm. Ampicillin (100 µg ml^{-1}) or gentamicin (10 µg ml^{-1}) were added to the media where appropriate.

Colony PCR amplification of *wza, wzb, and wzc* region. The modified EB6 forward (5'-GGTCAGGGATCCAACAGTCTG-3') and EB7 reverse (5'-TCGCGGATCCAATTGTT ACGA-3') primer sequences taken from Su *et al.* were used for genotypic confirmation (2). The primers were originally designed by Reid and Whitfield, to amplify the region spanning the *wza, wzb, wzc* cluster (1). The removal of three nucleotides from the 5' end of EB6 allowed for both EB6 and EB7 to have similar melting temperatures of 57.3°C and 56.6°C, respectively (2). The lyophilized primers ordered from Integrated DNA Technologies were resuspended in sterile distilled water to prepare stock solutions of 100 µM. Colony PCR reactions were performed in 50 µl volumes containing 5 µl of 10X PCR Buffer (- $MgCl_2$), 1.5 µl of 50 mM $MgCl_2$, 1.5 µl of 10 mM dNTP mix, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 0.2 µl of Invitrogen Platinum® Taq DNA Polymerase, a colony of the strains (Wild-type , CWG281 and DH5α+pUC19) and sterile water. A negative control was conducted without the addition of any bacterial cells, and the positive control used commercially available primers to amplify the pUC19 from DH5α transformants. Thermo-cycler conditions consisted of an initial denaturation at 94 °C for 120 seconds, then 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 50 °C for 30 seconds, and extension at

72 °C for 30 seconds, with a final cycle for extension of 10 minutes at 72°C. The PCR products were analyzed on a 0.8% agarose gel pre-stained with SYBR-Safe, ran at 120V for 1 hour. Amplicons were visualized by UV light using the BioRad ChemiDoc imaging system.

Erythromycin disc diffusion assay of *E. coli* K30 E69 and CWG281. Using a modified version of the Kirby-Bauer method, erythromycin disc diffusion assays were performed to confirm sensitivity/resistance phenotypes of *E. coli* K30 E69 and CWG281 (9). Uniform LB agar plates (0.4 cm in depth) were prepared by pipetting 17 ml of sterile media into petri dishes supplemented with antibiotic where appropriate. A colony of each strains was inoculated in 5 ml of LB media and were grown overnight at 37°C. On the following day, the cultures were normalized to $OD600_{nm}$ of ~1.0 through dilutions with sterile LB broth and use of the Spectronic 20+ spectrophotometer. 100 µL of each diluted culture was then spread onto LB agar plates to obtain a confluent lawn. After the spread plates fully dry, three 15 µg erythromycin discs (0.6 cm diameter) were placed at equal distances using sterilized forceps. The plates were incubated for ~48 hours at 37°C to accommodate slow growth of the CWG281 strain. The diameters of the zone of inhibitions, measured in centimeters, were defined by the visual characterization of an area of no cell growth surrounding the disc. A larger diameter of the zone of inhibition indicates higher susceptibility to erythromycin whereas a smaller size of the zone of inhibition indicates lower susceptibility.

Optimization of arabinose induction in CWG281+pBAD24-*wza*. To determine the optimum arabinose supplement necessary to induce pBAD24-*wza* expression in CWG281 transformants without substantially impairing its growth, various concentrations of L-arabinose were selected and prepared with sterile water: 0.005%, 0.008%, 0.1% and 1%. A similar procedure outlined for the erythromycin disc diffusion assay was followed. However, instead of the antibiotic discs, Whatman paper discs of the same size (0.6 cm) were used to add 10 µl of arabinose. We refer to this experiment as arabinose disc diffusion assay. One disc was placed on each half of the plate. A 10 µl volume of sterile water was added to two Whatman paper discs, serving as negative controls. The experiment were conducted on the following strains: CWG281+pBAD24-*wza*, CWG281+pBAD24-*GFP*, DH5α+pBAD24-*wza*, CWG281 and K30 E69. Plates were incubated for ~48 hours before checking for inhibition.

Arabinose-antibiotic disc diffusion assays of pBAD24-*wza* transformants using erythromycin discs supplemented with various arabinose concentrations. In order to minimize variability in experimentation and properly evaluate the effect of complementing *wza* in CWG281, the protocol for the erythromycin disc diffusion assay were further modified with the addition of 10 ul of 0.005%, 0.008%, 0.1% or 1% arabinose on top of the antibiotic discs (arabinose-antibiotic disc diffusion assay). In parallel, one set of erythromycin disc diffusion assay and a set for arabinose-only diffusion assay using Whatman paper discs were also conducted. The same list of strains above were used in this experiment with DH5α included, acting as control for DH5α+pBAD24-*wza*. Each experiment was conducted in three plates, with two or three discs per plate. The same quantitative analysis was done to measure the diameter of zones of inhibition.

RESULTS

Colony PCR confirmed the presence/knockout of *wza* in *E. coli* K30 E69 and CWG281, respectively. In order to verify if the strains K30 E69 and CWG281 correspond with the published genotypes, the region spanning the *wza-wzb-wzc* cluster was amplified using colony PCR. Agarose gel electrophoresis was used to resolve the PCR products (Fig. 2).

The DH5α+pUC19 positive control in lane 2 showed the expected band of ~2600 bp, corresponding to the size of pUC19. No bands were detected for the negative control (without the DNA template), as shown in lane 3. Lane 4 showed a band of~4200 bp, which was the expected amplicon size for the *wzy* locus in K30 E69 (2). For the CWG281 in lane 5, a slightly larger band of >4200 bp was observed due to the insertion of a gentamicin resistance cassette (616 bp) into *wza* (2).These results confirmed the genotype of our strains of interest.

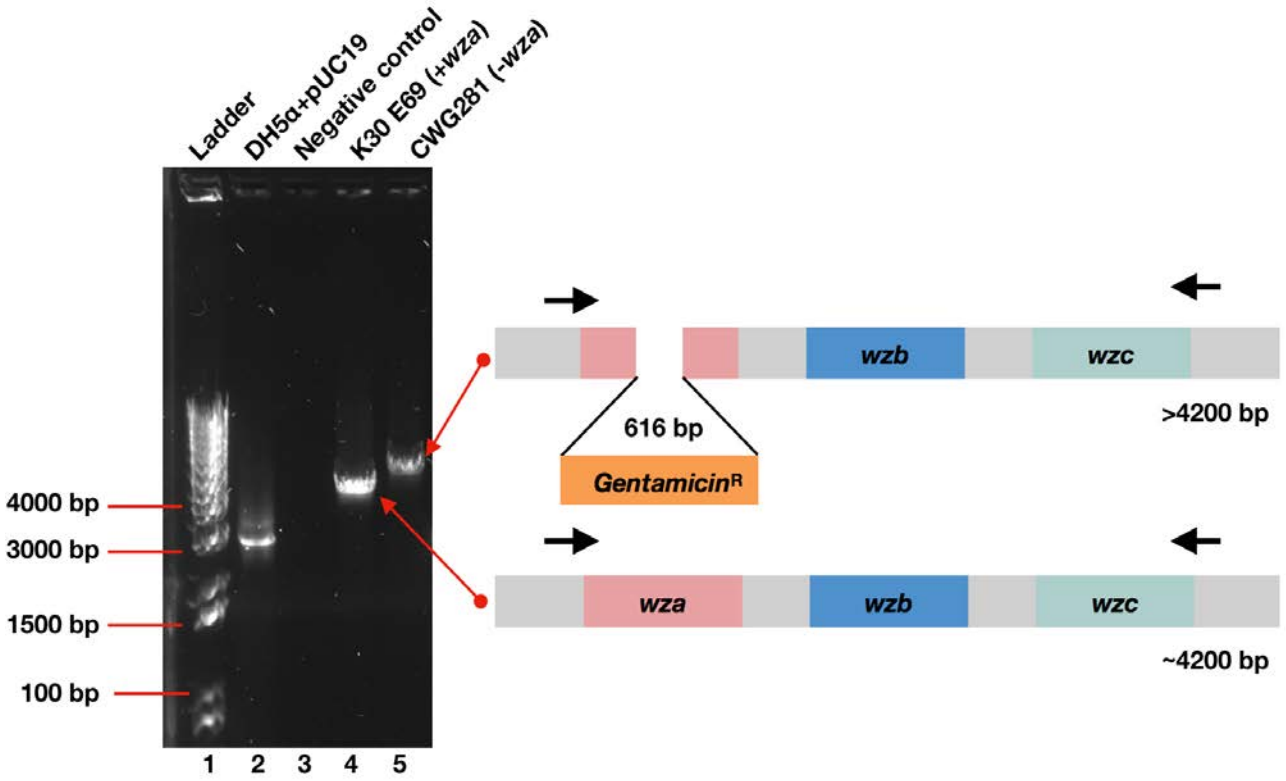

FIG. 2 Colony PCR amplification of *wza-wzb-wzc* cluster in K30 E69 and CWG281. The modified EB6 and ED7 primers (black arrows) were designed by Reid and Whitfield to amplify the region spanning *wzy* in the wild-type E69 and the *wza*-knockout CWG281. The expected band for the wild-type region should be approximately 4200 bp. Due to the insertion of the 616 bp gentamicin resistance cassette within *wza*, the CWG281 region should be larger than 4200 bp. Lane 1: 1 Kb Plus DNA ladder. Lane 2: DH5a+pUC19 positive control amplified with commercially available primers for pUC19; Lane 3: negative control with sterile distilled water; Lane 4: K30 E69; Lane 5: CWG281.

Erythromycin sensitivity/resistance in *E. coli* K30 E69 and CWG281 was confirmed using disc diffusion assay. To confirm the susceptibility of *E. coli* K30 E69 and resistance of CWG281 mutant to erythromycin, we conducted an erythromycin disc diffusion assay. The diameter of the zones of inhibition were measured and marked by the red dashed circles (Fig. 3B, C). In K30 E69, a clear zone of inhibition was observed, indicating the sensitivity of the WT strain to erythromycin. Conversely, a smaller zone was seen in CWG281 which confirmed its erythromycin-resistant phenotype (Fig. 3A). The average zone of inhibition for E69 was 2.2 cm (Fig. 3A, C), while CWG281 had an average zone of inhibition of 1.3 cm (Fig. 3B, C). Our results were consistent with previous studies (2, 3), and verified that the *wza*-knockout CWG281 confer resistance to erythromycin.

Arabinose induction of pBAD24-*wza* was not found to impair cell viability. *wza* overexpression was initially suggested to impair cell growth by Yuen *et al.* (7,12). Thus, finding the optimal concentration of arabinose to induce pBAD24-*wza* induction in CWG281 transformants without affecting its growth was necessary. The optimization experiment was conducted using arabinose disc diffusion assay on solid media. The WT, CWG281 and CWG281+pBAD24-*wza* strains showed comparable "zones of clearance" in the range of 0.6-0.8 cm (Fig. 4A), which was also comparable to a control disc with water only. Our results indicate that the tested arabinose range (0.005%-1%) does not impair cell viability.

Complementation of *wza* in *E. coli* CWG281 restored erythromycin sensitivity at 1% arabinose induction on solid media. To find the arabinose concentration required to add onto the erythromycin discs that leads to zones of inhibition similar to WT, we performed an arabinose-antibiotic disc diffusion assay, where we added different concentrations of

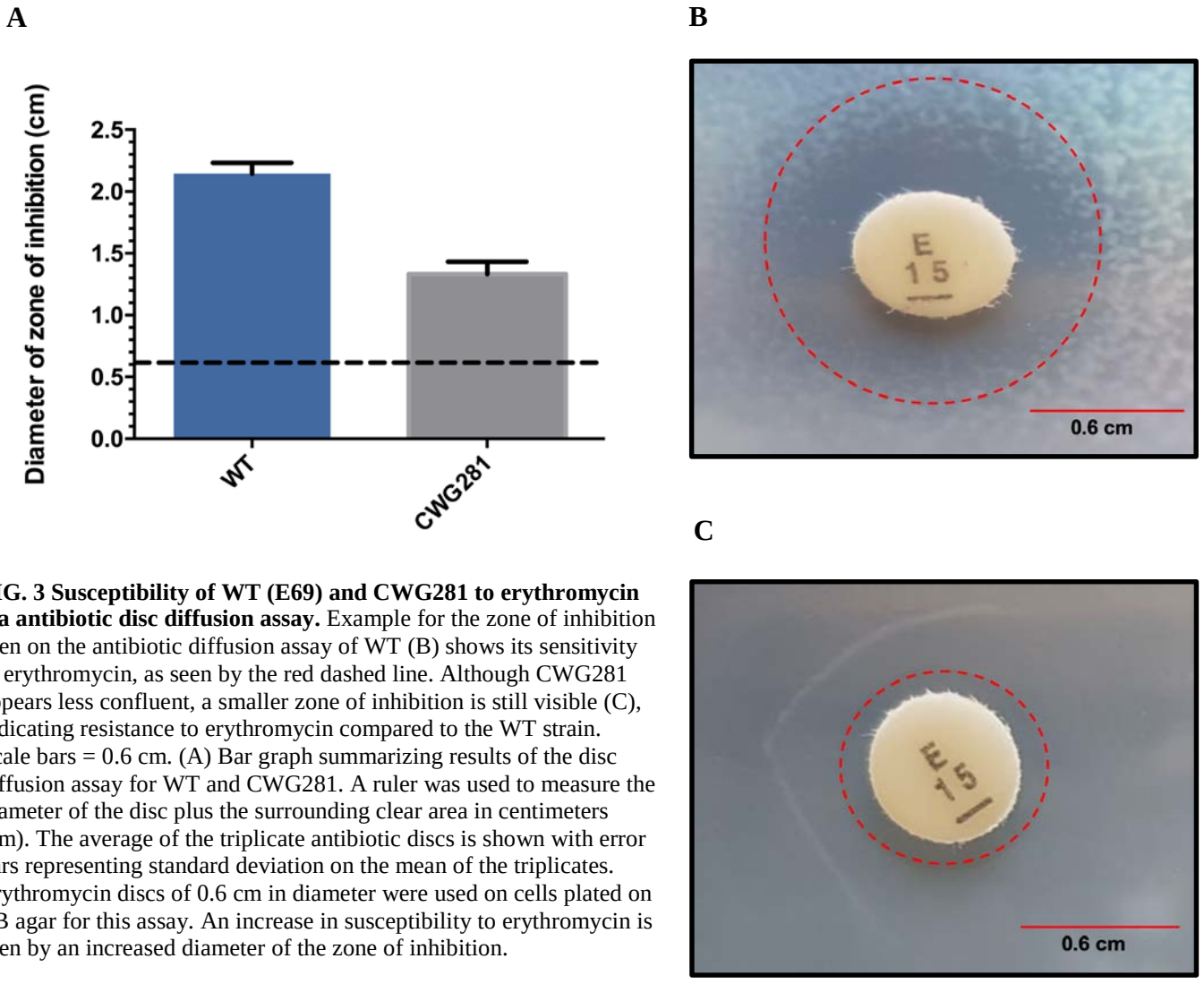

FIG. 3 Susceptibility of WT (E69) and CWG281 to erythromycin via antibiotic disc diffusion assay. Example for the zone of inhibition seen on the antibiotic diffusion assay of WT (B) shows its sensitivity to erythromycin, as seen by the red dashed line. Although CWG281 appears less confluent, a smaller zone of inhibition is still visible (C), indicating resistance to erythromycin compared to the WT strain. Scale bars = 0.6 cm. (A) Bar graph summarizing results of the disc diffusion assay for WT and CWG281. A ruler was used to measure the diameter of the disc plus the surrounding clear area in centimeters (cm). The average of the triplicate antibiotic discs is shown with error bars representing standard deviation on the mean of the triplicates. Erythromycin discs of 0.6 cm 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.

arabinose to erythromycin discs. A zone of inhibition comparable to the WT (positive control) indicates restoration of erythromycin sensitivity in CWG281+pBAD24-*wza*. One set of experiment contained erythromycin discs supplemented with 0.005%-1% arabinose, while another set had erythromycin discs only, acting as negative controls without plasmid induction. In Figure 4B, the WT showed zones of inhibition with an average diameter of ~1.2 cm. In comparison to the WT, we found a larger diameter (>1.2 cm) of the zones of inhibition in CWG281+pBAD24-*wza* and DH5α+pBAD-*wza* at 1% arabinose.

To increase the robustness of our findings, we repeated our arabinose-erythromycin disc diffusion assay in triplicate plates at 1% arabinose. We measured the zones of inhibition of the six different strains containing arabinose-only discs, erythromycin-only discs, as well as erythromycin discs supplemented with arabinose. All strains without pBAD24-*wza* demonstrated a consistent, baseline level of erythromycin resistance, showing zones of inhibition in the range of 1.0-1.3 cm in diameter. The transformed strain CWG281+pBAD24-*wza* had zones of inhibition of 2.5 cm in diameter, which were greater than that of the CWG281 and WT strains, as expected (Fig. 5). Interestingly, DH5α+pBAD-*wza* also showed larger zones of inhibition (~2 cm) in comparison to DH5α, suggesting an increase in erythromycin sensitivity (Fig. 5). Our results demonstrate that at 1% arabinose induction, plasmid-mediated complementation of *wza* in *E. coli* CWG281 restores erythromycin sensitivity.

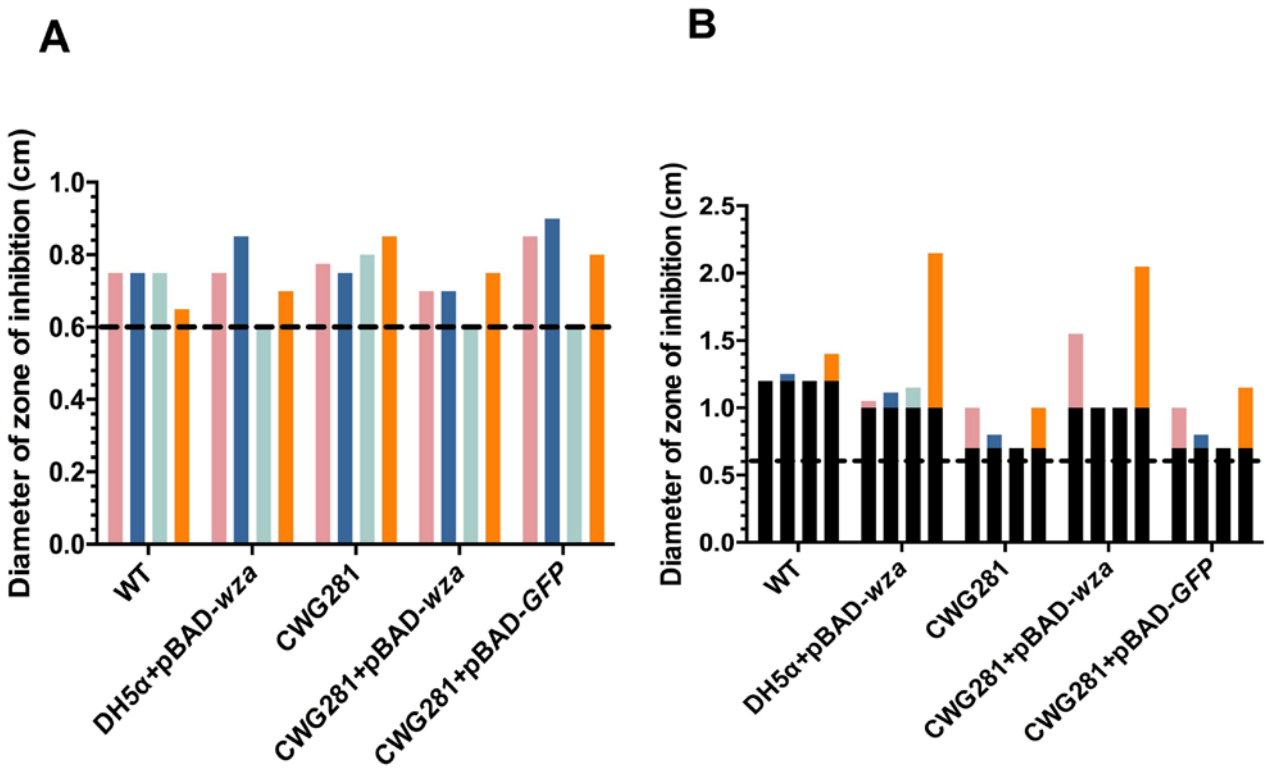

FIG. 4 Arabinose induction optimization of pBAD24-*wza* and selection of arabinose concentration that properly evaluates erythromycin sensitivity in CWG281+pBAD-*wza*. (A) Bar graph summarizing results of the arabinose disc diffusion assay for WT, DH5a+pBAD-*wza*, CWG281, CWG281+pBAD-*wza* and CWG281+pBAD-GFP. The arabinose disc diffusion assay was conducted on solid media to determine the optimal arabinose concentration for pBAD24-*wza* induction. Arabinose concentrations used to induce pBAD24-*wza* are represented by the different colored bars: Pink = 0.005%, Blue = 0.008%, Teal = 0.1%, orange = 1%. A ruler was used to measure the diameter of the disc plus the surrounding clear area in centimeters (cm). The height of the bars shows the average diameter of the zones of inhibition of the three plated arabinose discs. Black dashed lines represent diameter of discs. (B) Bar graph summarizing results of the modified arabinose-antibiotic disc diffusion assay with 15 ug erythromycin discs supplemented 0.005%-1% arabinose using the five strains listed above. The black bars represent the diameter of the zones of inhibition with erythromycin discs only. The colored bars on the top show the increase in diameter of inhibition zones upon arabinose addition (Pink = 0.005%, Blue = 0.008%, Teal = 0.1%, orange = 1%). An increase in susceptibility to erythromycin is seen by an increased diameter of the zone of inhibition.

DISCUSSION

The assembly and transport of type 1 capsular polysaccharide is mediated through the Wzy system (4). Previous studies have shown that deletion of Wza confers resistance to erythromycin (2). In this study, we further characterized the role of *wza* in *E. coli* K30, by examining the effect of *wza* complementation in a *wza* knockout strain CWG281. Observations from Pochanart *et al.* have suggested the route of entry of erythromycin may depend on Wza. The crystal structure of Wza has revealed that the channel is large enough to allow translocation of erythromycin molecules into the periplasm (2, 16). With this in mind, we hypothesized that plasmid-mediated complementation of *wza* will restore erythromycin sensitivity in CWG281.

To investigate this, we assessed the erythromycin sensitivity of the wild-type *E coli.* K30 E69 and the *wza*-knockout mutant CWG281, containing an arabinose-inducible pBAD24-*wza.* This transformed strain exhibited more erythromycin-sensitivity than the untransformed CWG281 and WT when the plasmid was induced with the addition of 1% arabinose, but not with 0.005%, 0.008% or 0.1% (Fig. 4B). Interestingly, DH5α+pBAD24-*wza*, originally included as a control, was also observed to have an increase in erythromycin sensitivity when compared to untransformed DH5α (Fig. 5).

Restoration of erythromycin sensitivity only occurred at 1% arabinose concentration in *E. coli* CWG281 on solid media. Pochanart *et al*'s study previously showed that complementation of *E. coli* with pBAD24-*wza* resulted in reduced growth rates with increasing concentrations of arabinose (7). This was observed in both liquid cultures and solid media prepared with arabinose (7). The authors proposed that the overexpression of *wza* may have resulted in reduced cell viability due to an overabundance of Wza channels embedded in the OM. This damages the OM barrier, leading to membrane instability (7). In addition, this observation is also consistent with previous studies, which have found cell toxicity and severely reduced growth rates directly related to overexpression of membrane proteins (15).

To test whether Wza expression is able to restore the erythromycin sensitive phenotype in *E. coli* K30, we first needed to establish a *wza* induction condition that does not affect cell viability. Otherwise, cell death caused by wza overexpression can not be discriminated from death due to erythromycin sensitivity. We developed a newly modified arabinose-antibiotic disc diffusion assay to conduct both the optimization of arabinose induction and erythromycin sensitivity on solid media. To do this, the arabinose was seeded into paper discs at concentrations of 0.005%, 0.008%, 0.1% and 1% separately. The various strains were grown in the presence of these arabinose discs. We did not observe zones of inhibition around the arabinose discs indicative of cell death (Fig. 4A). We next added arabinose to erythromycin discs and measured the zone of inhibition. We observed higher zones of inhibition in CWG281+pBAD24-*wza* in comparison to WT with the addition of 1% arabinose to the erythromycin discs, but not with 0.005%, 0.008% or 0.1% (Fig. 4B). These data suggest that expression of *wza* is sufficient to restore an erythromycin sensitive phenotype in *E. coli* K30 at 1% arabinose.

It is worth noting that our study used arabinose concentrations different than those used by Pochanart *et al.* They reported that partial to complete erythromycin sensitivity were restored at 0.006%, 0.008% and 0.01% arabinose (7). The difference is likely due to the different methodologies used to induce plasmid expression with arabinose. While Pochanart *et al* studied the *E. coli* growth in liquid culture or solid plates prepared with arabinose, we used paper discs and a radial diffusion assay (7). It is probable that the concentration of

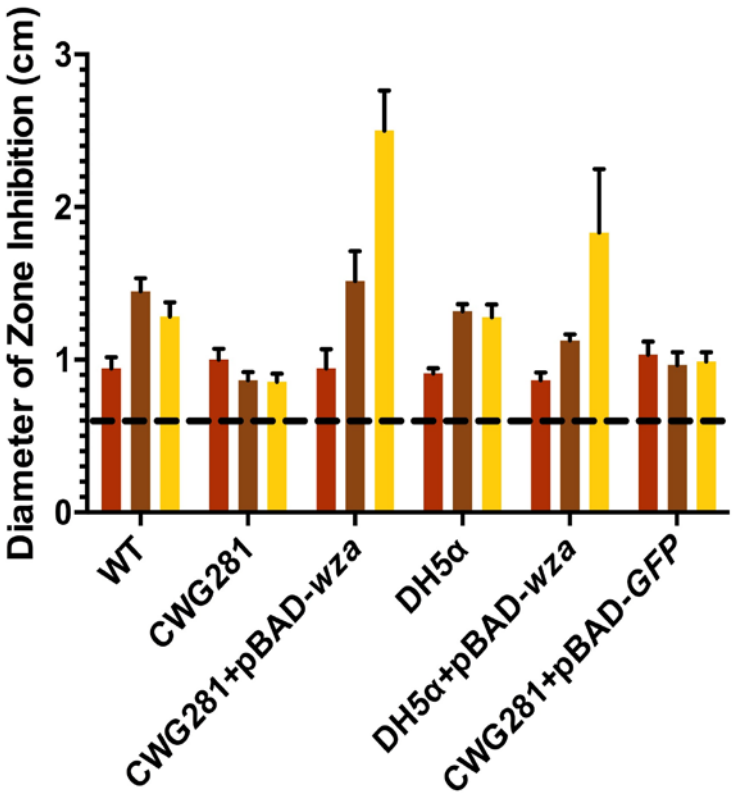

FIG. 5 Plasmid-mediated complementation of *wza* in *E. coli* CWG281 restores erythromycin sensitivity at 1% arabinose induction via arabinose-antibiotic disc diffusion assay. The bar graph shows the compiled measured zones of inhibition obtained from the arabinose-erythromycin disc diffusion assay for WT, CWG281, CWG281+pBAD-*wza*, DH5a, DH5a+pBAD-*wza* and CWG281+pBAD-GFP. Assays were conducted on LB or LB-Amp agar using 1% arabinose only discs (red), 15 ug erythromycin only discs (brown) and 15 ug erythromycin discs supplemented with 1% arabinose (yellow). A ruler was used to measure the diameter of the disc plus the surrounding clear area in centimeters (cm). The average diameter of the zones of inhibition of the three plated discs for each assay are represented by the bar heights. The errors base represent standard deviation on the mean of the triplicates. Black dashed lines represent diameter of discs used (0.6 cm). An increase in susceptibility to erythromycin is seen by an increased diameter of the zone of inhibition.

arabinose from the discs is different than the concentration of arabinose in liquid media or agar plates. We suspect that there is a limit on the amount of arabinose that can be diffused out of the discs. In our experiment, the actual amount of arabinose diffused from the discs containing 0.005%, 0.008% and 0.1% arabinose were possibly too low and did not induce enough *wza* expression to observe erythromycin sensitivity. Meanwhile, the disc containing 1% arabinose solution possibly have released enough inducers to achieve high enough pBAD-*wza* expression for observable erythromycin sensitivity restoration (Fig. 4B). This hypothesis may provide an answer to why Pochanart *et al.* observed restoration of erythromycin-sensitive phenotype with 0.008% arabinose, while we achieved the same observation at 1% arabinose.

To test our hypothesis, two experiments should be carried out in parallel to compare arabinose induction between the two different methods. The same concentrations of arabinose should be i) seeded onto the erythromycin discs and ii) supplemented into the solid media. of separate plates. The amount of arabinose incorporated into the media will remain constant, while the amount of arabinose that diffuses from the disc is unknown. Therefore, measuring the level of erythromycin sensitivity with erythromycin discs will confirm whether the plasmid induction level is indeed different between the two methods. A wider range of arabinose concentrations can also be tested.

Overexpression of Wza sensitizes DH5α to erythromycin. Interestingly, the strain we used as a control for cell growth, DH5α+pBAD-*wza*, exhibited increased erythromycin sensitivity compared to the negative control DH5α at 1% arabinose induction (Fig. 5). The difference between DH5α+pBAD24-*wza* and CWG281+ pBAD24-*wza* is that DH5α already expresses a normal level of *wza* as its genome has not been disrupted. Therefore, transforming the pBAD-*wza* into the strain further increases the number of Wza channels in the OM.

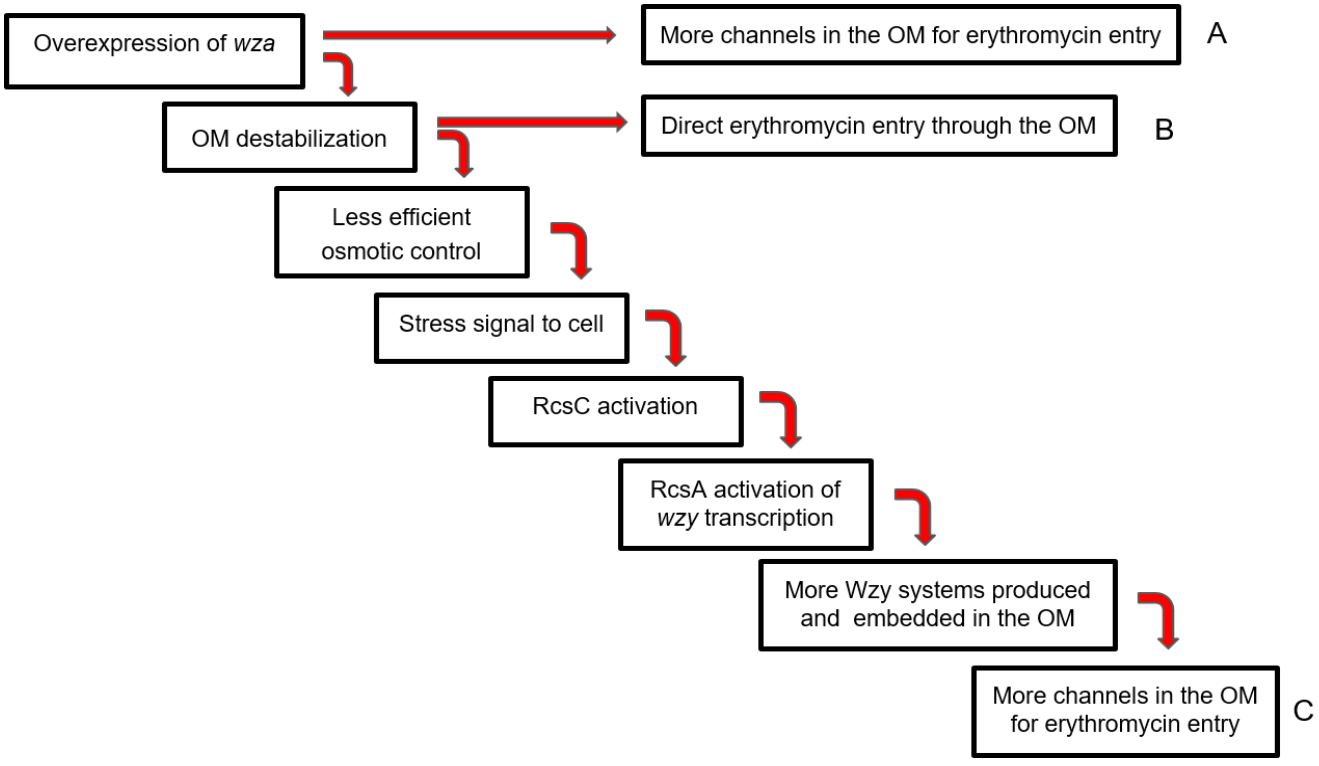

FIG. 6 Possible explanations for increased erythromycin sensitivity due to *wza* overexpression. This flowchart illustrates the three proposed models accounting for increased erythromycin sensitivity in *E. coli* due to *wza* overexpression. Model A indicates a direct relationship between the overexpression of *wza* leading to an increased number of channels in the OM for erythromycin entry. Model B implies that the overexpression of *wza* destabilizes the OM, resulting in increased membrane permeability for direct entry of erythromycin into the cell. Model C suggests that through model B, the destabilized OM leads to less efficient osmotic control. This sends a stress signal to the cell, which activates the Rcs phosphorelay system, resulting in signal relay from RcsC to RcsA. RcsA then activates the transcription of *wzy*, causing more Wzy systems to be embedded in the OM. As a result, the increased channels in the OM permits escalates erythromycin entry.

The increase in erythromycin sensitivity in DH5α+pBAD-*wza* is likely due to the an overexpression of the Wza channels (Fig. 6). The increase in Wza alone in the OM may account for higher erythromycin-sensitivity, due to the presence of more channels providing route of entry for erythromycin molecules. However, an alternative explanation proposed by Pochanart *et al* may also be possible (7). It has been suggested that the insertion of Wza into the OM forms pores in the OM (16). As a result, the overexpression of Wza leads to more pore formation in the OM (16). This in turn destabilizes the OM and leads to higher membrane permeability, which allows the passage of larger molecules, such as erythromycin, through the cell envelope without the need to go through the Wza channels (7) (Fig. 6).

In addition, an upregulation of Wzy system in the OM may also contribute to erythromycin sensitivity (Fig. 6). As mentioned previously, the overexpression of Wza channels in the OM may lead to membrane destabilization, resulting in cellular stress. Damage of the OM results in an increased passage of large molecules through the membrane and may offset the osmotic balance, resulting in osmotic stress (13) (Fig. 6). Studies have shown that cellular or osmotic stress induces the Rcs phosphorelay system, which are positive activators of the *wzy* gene cassette including *wza*, *wzb* and *wzc*, for capsular polysaccharide synthesis (18). The stress response pathway begins with signaling the sensor, RcsC, which relays the internal signal to RcsA that activates *wzy* transcription (19). The increased transcription of *wza*, *wzb* and *wzc* transcription leads to higher number of complete Wzy systems in the OM, which will allow the passage of more erythromycin molecules into the periplasm and leading to an increased sensitivity to erythromycin (Fig. 6). Therefore, the sensitization of DH5α to erythromycin may be attributed to both the translocation of antibiotic molecules through the increased number of Wza channels and Wzy systems, or direct passage through the cell envelope due to a destabilized OM.

Limitations The CWG281 cells used in our experiment exhibited slower and less confluent growth respectively in overnight cultures and on plates, which may be a sign of cellular stress (figure not shown). As the CWG281 cells were carried over from the Ponchanart *et al.* study, we speculate that the cells may have been passaged too many times, experienced too many freeze-thaw cycles and became less viable.

Future Directions In CWG281, the loss of Wza confers resistance to erythromycin (2,7). In this study, it was observed that the complementation of *wza* in CWG281 results in the restoration of erythromycin sensitivity. The plasmid carrying the *wza* gene may have led to the expression of Wza in the OM, providing a route of entry for erythromycin (2, 7) (Fig. 1B). However, there might be other mechanisms by which *wza* expression influences erythromycin sensitivity. For example, the OM may be destabilized due to Wza overexpression, leading to the passage of large molecules, such as antibiotics, into the cytosol (13). A future study investigating the mechanism of restoration of erythromycin-sensitivity can involve destabilizing the OM in CWG281 in the absence of *wza* expression. A possible method is the overexpression of a different outer membrane pore protein, such as OmpC/OmpF or LamB (Maltoporin), to determine if outer membrane destabilization by proteins is sufficient to sensitize cells to erythromycin (20). Similar to our experimental approach, a modified arabinose-antibiotic disc diffusion assay can be used to observe the effects on erythromycin sensitivity, with CWG281 (negative control), CWG281 with overexpressed OM proteins, and CWG281+pBAD24-*wza* (positive control). If the CWG281 strain with overexpressed OM proteins exhibits comparable level of erythromycin sensitivity as CWG281+pBAD24-*wza*, the result likely suggests that destabilized membrane from *wza* overexpression provides an entry for erythromycin into the cell.

An alternative approach to verify if erythromycin translocates through the Wza channel into the cell is to block the Wza channel using an inhibitor. The most effective inhibitor is an eight-membered cyclic glucan, octakis, which has an equivalent diameter as the Wza pore and binds to the α-helix barrel inside the channel (21). WT *E. coli* K30 E69 can be treated with octakis to inhibit present Wza channels. A modified antibiotic disc diffusion assay can be used to assess the level of erythromycin sensitivity in the treated WT, using untreated *E. coli* K30 E69 and CWG281 as a positive and negative control, respectively. We have already previously established that untreated WT exhibit greater erythromycin sensitivity than

CWG281. If the octakis treated WT is observed to have the same-sized zone of inhibition as CWG281, it is likely that the inhibited Wza channel prevents erythromycin entry. Thus, suggesting that the Wza channel directly provides a route of entry for erythromycin.

Additionally, the increase in Wza channels could destabilize the outer membrane and induce a stress response that triggers *wzb* and *wzc* transcription, forming higher number of complete Wzy systems in the OM (19). Studies have shown that cellular stress can signal the sensor, RcsC, which triggers a replay of internal signal to RcsA that activates *wzy* expression (19). In addition, our erythromycin sensitivity assay may be repeated on a pBAD24-*wza* transformed *rcsA* mutant strain of *E. coli*, in order to assess whether the overexpression of *wza* activates the Rcs phosphorelay system. In order to investigate the effect of *wza* expression on the upregulation of *wzb* and *wzc* transcription, future studies may evaluate the mRNA levels of *wzb* and *wzc* using Reverse Transcription-quantitative PCR (RT-qPCR). To have a better understanding of the obtained results in this study, it would be key to understand the effects of the induction of *wza* expression on other cellular mechanisms that might lead to the increase in erythromycin sensitivity.

Future Studies may also wish to test plasmid-mediated complementation of *wzb* or *wzc* into their respective knockout mutants CWG343 or CWG285 (1). This would inform whether these components of the *wzy* system also play a role in restoring erythromycin sensitivity.

Further, it has been shown by previous studies that the deletion of *wza* also leads to resistance to clarithromycin (6). Future studies may investigate whether the complementation with *wza* would also lead to the restoration of clarithromycin sensitivity.

Conclusion In conclusion, we have shown that induction of pBAD24-*wza* at 1% arabinose results in plasmid-mediated complementation of a *wza* knockout mutant *E. coli* CWG281. Through our arabinose-antibiotic disc diffusion assay, we are able to appropriately evaluate the restoration of erythromycin susceptibility and the sensitization of erythromycin conferred by an overexpression of *wza* in CWG281 and DH5α, respectively, This study suggests two conclusions. First, the complementation of *wza* in *E. coli* CWG281 is sufficient to restore an erythromycin-sensitive phenotype supporting our hypothesis that Wza plays a role in erythromycin sensitivity. Second, the overexpression of *wza* can increase erythromycin sensitivity in bacteria containing functional Wzy system.

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REFERENCES

1. Reid, AN, Whitfield, C. 2005. Functional analysis of conserved gene products involved in assembly of *Escherichia coli* capsules and exopolysaccharides: Evidence for molecular recognition between Wza and Wzc for colanic acid biosynthesis. J. Bacteriol. 187:5470-5481.
2. Su, A, Wang, A, Yeo, L. 2015. Deletion of group 1 capsular gene *wza* in *Escherichia coli* E69 confers resistance to the antibiotic erythromycin on solid media but not liquid media. JEMI. 3:1-8.
3. Ganal, S, Gaudin, C, Roensch, K, Tran, M. 2007. Effects of streptomycin and kanamycin on the production of capsular polysaccharides in *Escherichia coli* B23 cells. JEMI. 11:54-59.
4. Liu, H, Zhu, M, Zhu, S. 2011. Persistence of antibiotic resistance and capsule in *Escherichia coli* B23 after removal from sublethal kanamycin treatment. JEMI. 15:43-46.
5. Parmar, S, Rajwani, A, Sekhon, S, Suri, K. 2014. The *Escherichia coli* K12 capsule does not confer resistance to either tetracycline or streptomycin. JEMI. 18:76-81.
6. Chiu J, Han G, McCrystal K, Zuo M. 2017. Macrolide structure can confer differential susceptibility in *Escherichia coli* K30 deletions of group 1 capsule assembly genes. JEMI.
7. Pochanart, A, Richardson, M, Truong, P, Wang, J. 2017. Plasmid mediated complementation of *wza in Escherichia coli* K30 strain CWG281 restores erythromycin sensitivity. JEMI. In-press.
8. Delcour, AH. 2009. Outer membrane permeability and antibiotic resistance. Biochimica Et Biophysica Acta (BBA)-Proteins and Proteomics. 1794:808-816.

9. Botros, S, Mitchell, D, Van Ommen, C. 2015. Deletion of the *Escherichia coli* K30 group I capsule biosynthesis genes *wza*, *wzb* and *wzc* confers capsule-independent resistance to macrolide antibiotics. JEMI. 19:1-8
10. Jazdarehee, A, Anderson, JJ, Morrison, D, Pardoe, W. 2017. Deletion of *Escherichia coli* K30 type I capsule assembly gene *wzc* confers resistance to the antibiotic erythromycin in solid media. JEMI. 21:108-112.
11. Rana, G, Jang, Y, Ahn, P, Nan, J. 2016. Single Deletion of *Escherichia coli* K30 Group I Capsule Biosynthesis System Component, *wzb*, Is Not Sufficient to Confer Capsule-Independent Resistance to Erythromycin. JEMI. 20:19-24.
12. Yuen, B, Ting, J, Kang, K, Wong, T. 2017. Investigation of Wza in erythromycin sensitivity of *Escherichia coli* K30 E69 by genetic complementation. JEMI. 21:52-57.
13. Epand, RM, Walker, C, Epand, RF, Magarvey, NA. 2016. Molecular mechanisms of membrane targeting antibiotics. Biochimica Et Biophysica Acta Biomembranes. 1858:980-987.
14. Fujikawa, H, Morozumi, S. 2007. Modeling Surface Growth of *Escherichia coli* on Agar Plates. Appl. Environ. Microbiol. 73:2404
15. Wagner, S, Baars, L, Ytterberg, AJ, Klussmeier, A, Wagner, CS, Nord, O, Nygren, P, van Wijk, KJ, de Gier, J. 2007. Consequences of membrane protein overexpression in *Escherichia coli*. Molecular & Cellular Proteomics. 6:1527-1550.
16. Dong, C, Beis, K, Nesper, J, Brunkan, AL, Clarke, BR, Whitfield, C, Naismith, JH. 2006. The structure of Wza, the translocon for group 1 capsular polysaccharides in *Escherichia coli*, identifies a new class of outer membrane protein. Nature. 444:226.
17. Whitfield, C, Roberts, IS. 1999. Structure, assembly and regulation of expression of capsules in *Escherichia coli*. Mol. Microbiol. 31:1307-1319.
18. Sledjeski, DD, Gottesman, S. 1996. Osmotic shock induction of capsule synthesis in *Escherichia coli* K-12. J Bacteriol. 174:1204-1206.
19. Ebel, W, Trempy, JE. 1999. *Escherichia coli* RcsA, a positive activator of colanic acid capsular polysaccharide synthesis, functions to activate its own expression. J. Bacteriol. 181:577-584.
20. Molly, MP, Herbert, BR, Slade, MB, Rabilloud, T, Nouwens, AS, Williams, KL, Gooley, AA. 2000. Proteomic analysis of the *Escherichia coli* outer membrane. Eur. J. Biochem. 267: 2871-2881.
21. O'neill, EC, Field, RA. 2013. Antibiotics: Blocking bacterial defences. Nature Chemistry. **5:**642.