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

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SUMMARY In *Escherichia coli* serotype K30, the outer membrane channel protein Wza, involved in capsule secretion, has been genetically linked to macrolide sensitivity. Deletion of *wza* in erythromycin-sensitive *E. coli* K30 confers resistance to erythromycin. In this study, we tested whether complementation of *wza* would restore sensitivity to erythromycin in the *wza* knockout mutant *E. coli* CWG281. *wza* was cloned into the arabinose-inducible pBAD24 expression vector using Gibson assembly and then transformed into *E. coli* CWG281. Induction of pBAD24-wza_{K30} with 0.008% arabinose was observed to restore erythromycin sensitivity using Kirby-Bauer disk diffusion assays. Interestingly, induction of pBAD24-wza_{K30} with 1% arabinose was found to impair growth of *E. coli* CWG281 transformants. From this study, we conclude that complementation of *wza* in *E. coli* CWG281 is sufficient to restore erythromycin sensitivity. We further conclude that overexpression of *wza* in *E. coli* CWG281 impairs cell growth.

INTRODUCTION

Capsular polysaccharides (CPS), or K-antigens, are a major virulence factor in *Escherichia coli* that facilitate immune evasion and host tissue adherence (2). Group 1 capsules in particular contribute to pathogenesis in humans and animals by inhibiting the binding of opsonins, masking ligands necessary for phagocyte attachment, and participating in biofilm formation (1, 2). Furthermore, Group 1 capsules provide resistance to antimicrobial compounds by preventing these molecules from penetrating into the cell (1). Sublethal doses of kanamycin and streptomycin in *E. coli* induce increased CPS production, inhibiting antibiotic entry (1, 3). Group 1 capsule-dependent antibiotic resistance is however dependent on the class of antibiotic. *E. coli* serotype K30 is sensitive to macrolide antibiotics despite synthesizing group 1 *K*-antigens (4).

Group 1 (colanic acid) capsules are produced in a Wzy-dependent biosynthesis pathway (1, 2, 15). Several proteins are involved in group 1 CPS synthesis and exportation (Figure 1): the transferase WbaP, the flippase Wzx, the polymerase Wzy, the inner membrane tyrosine autokinase Wzc, the phosphotyrosine phosphatase Wzb, and the outer membrane translocon Wza (1, 15).

Macrolides, such as erythromycin, are a class of hydrophobic antibiotics that inhibit bacterial protein synthesis in the cytosol by binding to the 50S ribosomal subunit (5). Due to their large size and hydrophobicity, macrolides are largely ineffective against most strains of Gram-negative bacteria, including *E. coli*. Translocation of macrolides is restricted by the outer membrane (OM), which acts as a permeability barrier (5). In contrast to the inner membrane, the OM is comprised of two distinct leaflets: the outer leaflet containing LPS and the inner leaflet containing phospholipids (5). Strong lateral interactions exist between LPS molecules, due in part to the high density of negative charge in the LPS R-core (5). Divalent cations present in the environment bind to the anionic R-cores, neutralizing charge as well as linking adjacent LPS molecules (5). This results in a strong, stabilizing effect, making the OM less fluid and less permeable to diffusion than the inner membrane (5). As such, large hydrophobic molecules like macrolides are excluded from passage across the OM as disruption of core region interactions is thermodynamically unfavourable (5). The Gram-negative OM thus confers intrinsic resistance to macrolides in most *E. coli* strains (5).

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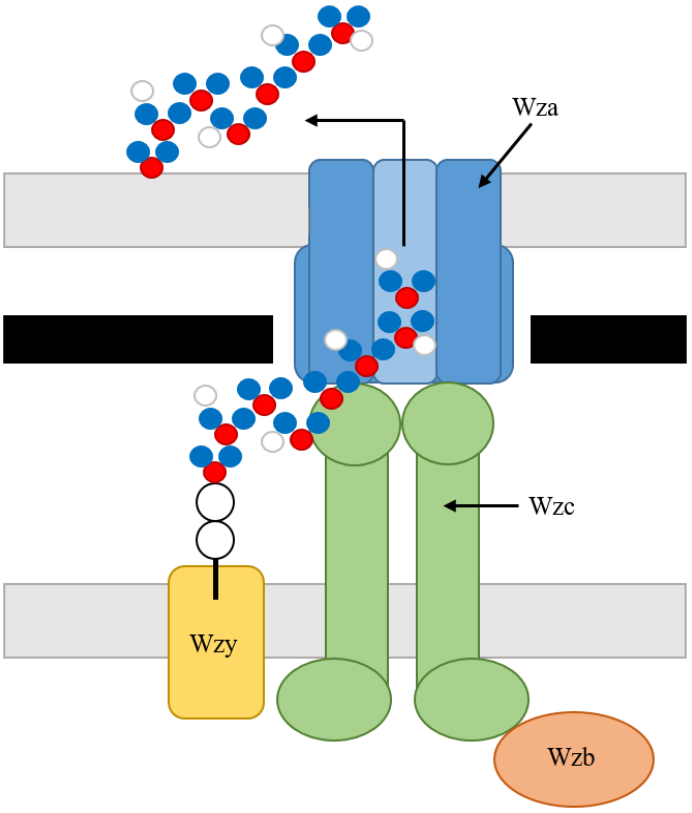


FIG. 1 Simplified model of Wzy-dependent group 1 capsule synthesis and export. Wzy polymerizes CPS subunits. Wzc and Wza interact to transport CPS from the periplasmic face of the inner membrane to the cell surface. Wzb is a cytosolic phosphatase that associates with Wzc.

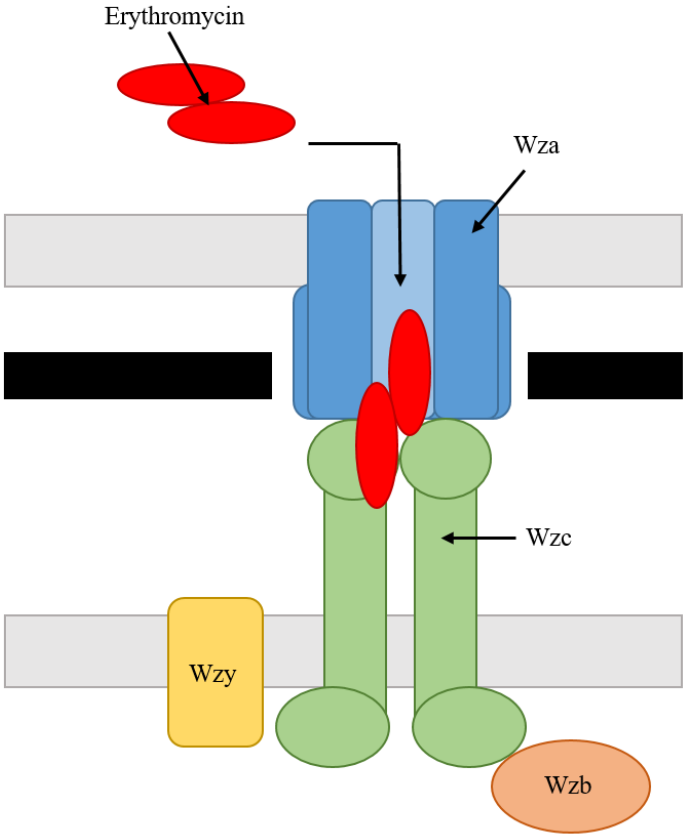


FIG. 2 Proposed model for erythromycin transport across the outer membrane. Erythromycin passes through the Wza channel. Interaction between Wza and Wzc allows for transport of erythromycin across the outer membrane and subsequently into the cytosol.

In erythromycin-sensitive *E. coli* K30, erythromycin transport across the OM is proposed to be facilitated by components of the Wzy-dependent CPS biosynthesis pathway, specifically Wza and Wzc (7. 8). In CPS export, the integral inner membrane protein Wzc, interacts with the OM translocon Wza to transport synthesized CPS polymers across the periplasm and onto the cell surface (6). Macrolides are proposed to pass through the Wza channel in the reverse direction (7, 8). Although this model has yet to be confirmed (Figure 2), single deletions of *wza* and *wzc* in *E. coli* K30 strain E69 confer resistance to erythromycin on solid media, suggesting that both Wza and Wzc are necessary for erythromycin import (7, 8). Indeed, although the Wza cavity is open at the extracellular face, it is closed at the periplasmic end (13). Wza must undergo a conformational change through interaction at the periplasmic domain with Wzc to form a channel, permitting export and entry of macromolecules (13). The structural model of Wza 2J58 shows a pore 17Å in diameter, exceeding the estimated size of erythromycin (11.97Å by 10.64Å), and could therefore accommodate entry of the antibiotic (7). Structural characterization of Wza reveals a unique alpha-helical structure, contrasting from the classical beta barrel topology of most OM proteins (14). The transmembrane domain of Wza is composed of 8 alpha helices, forming an octameric, ring-like channel structure (14). In Wza, the residues enclosing the pore are predominantly polar: a caveat to the model of erythromycin transport previously suggested (14). The hydrophobic nature of erythromycin may adversely affect its movement through the hydrophilic Wza channel (7). Due to these conflicting observations, further characterization of Wza is needed to further elucidate its role in erythromycin transport.

In order to further characterize the dependence of Wza in erythromycin sensitivity, Yuen *et al.* conducted a complementation study, cloning *wza* into the pCR®2.1-TOPO® vector and transforming the construct into the *wza* deletion mutant *E. coli* CWG281 (9). Yuen *et al.* did not observe restored erythromycin sensitivity, however, a lack of *wza* expression was proposed to account for this result (9). All plasmids generated by Yuen *et al.* carried *wza* inserts in the reverse orientation relative to the lac promoter suggesting that the forward orientation may have generated a toxic gene product and lethal phenotype (9, 10). It is also unclear whether the Yuen *et al.* construct contained the native *wza* promoter, as only 200 base pairs upstream of the methionine translational start site were included in the construct. The location of the *wza* promoter is unknown and may be located further upstream (9).

In this study, we attempted to complement *wza* in *E. coli* CWG281 using the arabinose-inducible pBAD24 expression vector. As *wza* overexpression was implicated to be lethal by Yuen *et al.*, an inducible expression vector allowing for tight control of *wza* expression levels was thought to be suitable (9). The pBAD system allows for tightly regulated protein expression through addition of carbon sources to growth media: L-glucose acts as a repressor while L-arabinose acts as an inducer (11). The pBAD vector was previously used by Reid *et al.* to restore CPS production in *E. coli* CWG281 (2). Expression of *wza* from the Reid *et al.* construct was not found to be lethal, further supporting the use of pBAD24 in this study (2).

We hypothesized that complementation of *wza* in *E. coli* CWG281, using a pBAD24-wza_{K30} construct, would restore erythromycin sensitivity. Expression of *wza* from pBAD24-wza_{K30} will allow for translocation of erythromycin across the OM barrier and subsequently into the cytosol, where the macrolide can act on the 50S ribosomal subunit (5, 7, 8). To investigate this, Kirby-Bauer disk diffusion assays were used to evaluate the erythromycin sensitivity of transformants. In parallel, bacterial growth of CWG281+pBAD24-wza_{K30} was monitored using optical density data at 600 nm (OD600). Our results suggest that erythromycin sensitivity and bacteria viability in CWG281+pBAD24-wza_{K30} is dependent on the level of uniformity in induction from pBAD24-wza_{K30}.

METHODS AND MATERIALS

Bacterial strains and growth conditions. *E. coli* K30 E69 (WT) (serotype: O9a:K30:H12) and CWG281 (*wza*$_{22min}$::*aadAwza*$_{K30}$::*aacC1*) strains were obtained from the laboratory of Dr. Chris Whitfield at the University of Guelph. *E. coli* K30 E69 is the isogenic parental strain of CWG281. Bacterial strains were isolated for use on Lysogeny broth (LB) agar plates and were grown in LB broth (3). pBAD24-wza_{K30} and pBAD24-GFP transformants generated in this study were grown in LB media containing 100 μg/ml ampicillin (LB-Amp).

Table 1 Primers used for PCR amplification of *wza* in this study.

Purpose	Direction	Sequence (5' to 3')[1]	T_m (°C)	Amplicon size (bp)
Genotypic confirmation	Forward	AACGTGGTAGAACTCCCGGATAG CGAC	65	747
	Reverse	TACCAAGCGCCTCTGCAAGGGTC ATAC	66	
Gibson assembly	Forward	tgggctagcaggaggGACATTATGAAGA AAAAACTTGTTAG	58	1176
	Reverse	gcttgcatgcctgcaTTAGTTTGGCCATCT CTTAATG	59	

Oligonucleotide primer design. All primers for polymerase chain reaction (PCR) amplification used in this study were designed using the E. coli K30 capsule biosynthesis cluster sequence (accession no. AF104912.3) and Snapgene Viewer software, and ordered from Integrated DNA Technologies (IDT), as shown in Table 1.

Colony PCR amplification of *wza* and agarose gel electrophoresis for genotypic confirmation of *wza* in *E. coli* K30 E69 and CWG281. In each 25 µl PCR reaction, a final concentration of 1X PCR Buffer - Mg, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.2 µM forward primer, 0.2 µM reverse primer, 0.1 µl Platinum® Taq DNA polymerase (Invitrogen) was used (16). Colony PCR was performed on *E. coli* K30 E69 and CWG281 by adding cells directly to tubes containing PCR reagents. Thermocycler conditions consisted of an initial denaturation at 94 °C for 2 minutes, then 30 cycles of denaturation at 94 °C for 10 seconds, annealing at 59 °C for 30 seconds, and extension at 72 °C for 60 seconds. This was followed by a final extension at 72 °C for 5 minutes. PCR products were run on a 1% agarose gel stained with 0.5 µg/ml ethidium bromide at 100V for 1 hour. Amplicons were visualized and imaged by UV light using the Alpha Innotech Alphaimager.

CWG281 erythromycin resistance confirmation test using the Kirby-Bauer disk diffusion assay. To confirm sensitivity/resistance phenotypes of *E. coli* K30 E69 and CWG281, the modified Kirby-Bauer disk diffusion assay outlined by Botros *et al.* was used (4). Uniform LB agar plates were made by pipetting 20 ml of autoclaved media into petri dishes. Overnight cultures of K30 E69 and CWG281, grown at 37℃, were diluted to an OD600 of 1.0. For each diluted culture, 100 µl was spread plated onto a uniform LB plate and left to fully dry: three replicates (plates) were spread for each of K30 E69 and CWG281. Sterilized forceps were then used to place two 15 µg erythromycin disks on each plate, one disk for each half of the plate. Plates were incubated for 48 hours at 37°C before checking for any zones of inhibition.

Gradient PCR amplification of *wza* for Gibson Assembly. *E. coli* K30 E69 genomic DNA was extracted using the PureLink® Genomic DNA kit (Invitrogen) and eluted in Tris-EDTA buffer. DNA concentration was quantified using the Nanodrop3000 spectrophotometer. PCR reactions were performed in 50 µl volumes containing 5 µl of 10X *Pfx* amplification buffer, 1 µl of 50 mM $MgSO_4$, 1.5 µl of 10 mM dNTP mix, 1.5 µl of 10 µM forward primer, 1.5 µl of 10 µM reverse primer, 0.4 µl of Platinum® *Pfx* DNA Polymerase (Invitrogen), 0.5 µl of extracted K30 genomic DNA and 38.6 µl of sterile water (17). Positive controls using 1.5 µl pUC19 forward primer, 1.5 pUC19 µl reverse primer and 1 µl pUC19 template DNA were also included. Gradient PCR thermocycling conditions consisted of an initial denaturation at 94 °C for 30 seconds, then 30 cycles of denaturation at 94 °C for 15 seconds, annealing at 52-60°C for 30 seconds, and extension at 68 °C for 90 seconds. This was followed by a final extension at 68°C for 10 minutes.

Cloning of pBAD24-*wza*$_{K30}$ plasmid. pBAD24 plasmid DNA was extracted using the PureLink® Quick Plasmid DNA Miniprep Kit (Invitrogen) and linearized with 1X NEBuffer 3.1, EcoRI, and PstI. A 1-hour incubation at 37°C was used. The *wza* amplicon and EcoRI/PstI-digested pBAD24 plasmid were purified and *wza* was ligated into the linearized pBAD24 vector using the Gibson Assembly® Cloning Kit (NEB), generating pBAD24-*wza*$_{K30}$. A 20 min reaction time at 50°C was used.

Preparation of chemically competent cells *E. coli* DH5α and CWG281 cells. A 1 mL overnight culture of each *E. coli* DH5α and CWG281 was inoculated into 200 ml of LB, and the diluted cultures were grown at 37°C with shaking until 0.3 - 0.7 OD600 was achieved. The cells were centrifuged at 3000 g for 15 minutes at 4°C and supernatant was discarded. The cell pellets were resuspended in 72 ml of chilled 100 mM $MgCl_2$ and spun at 2000 g for 15 minutes at 4°C, and supernatant was discarded. Subsequently, the cell pellets were resuspended in 40 ml of chilled 100 mM $CaCl_2$ and chilled on ice for 20 minutes before being spun at 2000 g for 15 minutes at 4°C. The supernatant was removed, and cell pellets were resuspended in 5 ml of chilled 85 mM $CaCl_2$ with 15% glycerol and spun at 1000 g for 15 minutes at 4°C. The supernatant was discarded, and the cell pellets were resuspended in 200 µl of chilled 85 mM $CaCl_2$ with 15% glycerol. 50 µl of DH5α and CWG281 competent cells was aliquoted into Eppendorf tubes and stored at -20°C (18).

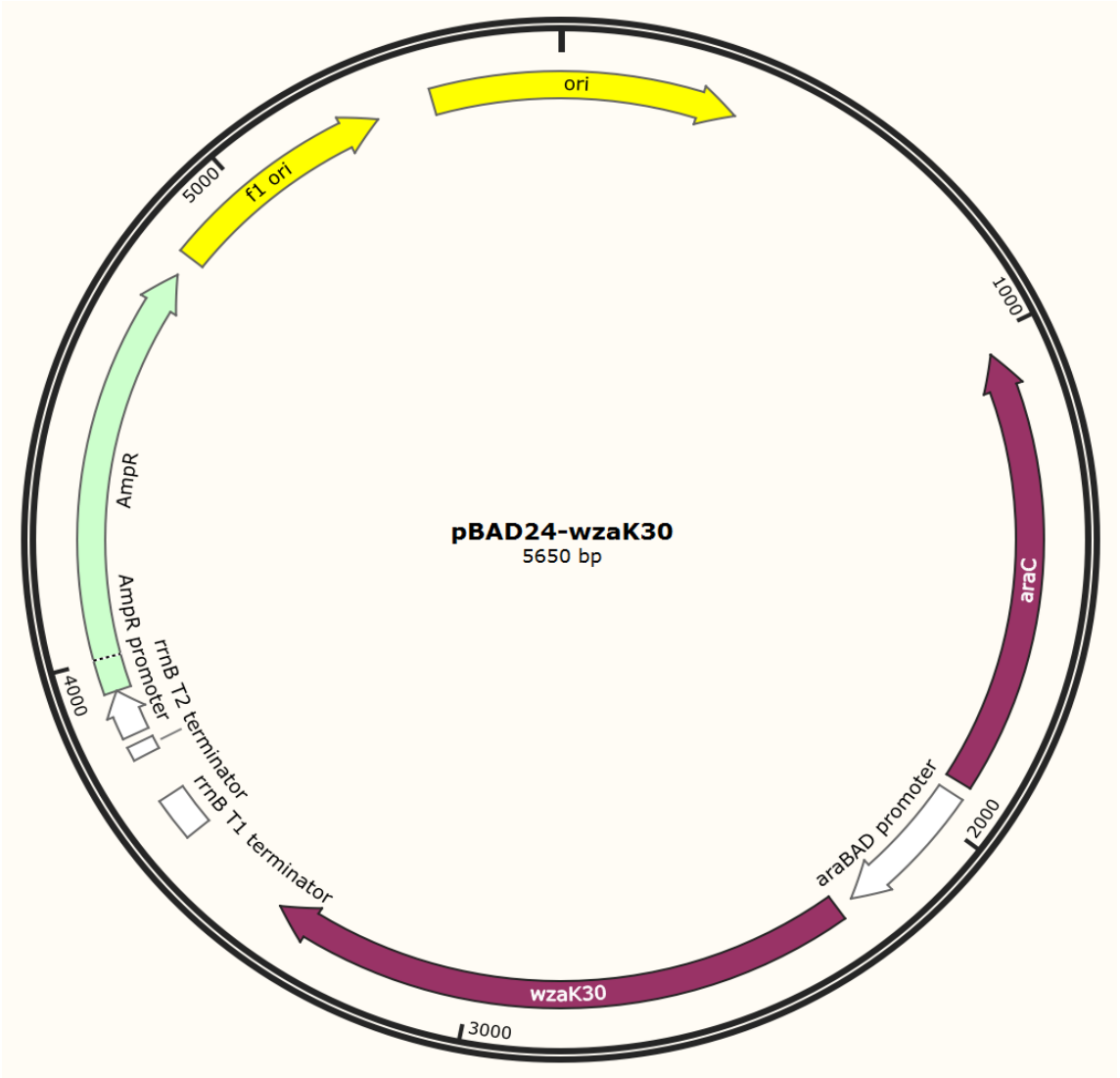


FIG. 3 Graphical representation of pBAD24-*wza*$_{K30}$ construct. Origins of replication (yellow), the *amp*^{R} gene (green), the *araC* gene (purple), the araBAD promoter (white), the cloned *wza* gene (purple), and terminator sequences (white) are shown.

Transformation of pBAD24-*wza*$_{K30}$ into chemically competent *E. coli* DH5α or CWG281 cells. One 2 µl aliquot of pBAD24-*wza*$_{K30}$ was added to a thawed tube of competent DH5α or CWG281 cells and subjected to heat shock at 42°C for 30 seconds. Each tube was chilled for 30 minutes on ice, followed by a 1-minute heat shock at 42°C. Subsequently, 250 µl of prewarmed LB was added to each tube. The tubes were incubated for 1 hour at 37°C. 100 µl of each transformation reaction was then plated on LB-Amp plates supplemented with 0.2% glucose. The plates were incubated at 37°C for 24 hours (19).

Isolation of pBAD24-*wza*$_{K30}$ and sequencing by Genewiz. Each of the 7 resulting DH5α+pBAD24-*wza*$_{K30}$ transformants were inoculated into 5 ml of LB supplemented with 100 µg/ml ampicillin and 0.2% glucose, and were grown overnight at 37°C. Three transformants were selected for plasmid isolation. Corresponding overnight cultures were centrifuged at 10, 000 g for 2 minutes. The supernatants were discarded and cell pellets were used for plasmid isolation using the PureLink ® Quick Plasmid DNA Miniprep Kit (Invitrogen). Plasmid DNA concentration was measured using the Nanodrop3000 spectrophotometer to ensure plasmid concentrations were sufficient for sequencing by Genewiz. Two 10 µl aliquots of each of the three plasmid preparations were placed into an 8-strip PCR tube. One of the plasmid aliquots was designated for sequencing in the forward direction and the other for the reverse direction. The 8-strip PCR tube was sent for sequencing at Genewiz.

Growth Curve. Overnight cultures of *E. coli* CWG281+pBAD24-*wza*$_{K30}$ and CWG281+pBAD24-GFP were diluted such that 1 ml at 1.0 OD600 was obtained. 500 µl of each diluted culture was added to 7 ml of LB-Amp media with 1% L-arabinose, and 7 ml of LB-Amp media with 1% L-glucose. Tubes were then incubated on a shaking platform at 37˚C. The optical density at 600 nm (OD600) was monitored at 30-minute intervals using a 96-well plate reader (Biotek) (9, 11).

Kirby-Bauer disk diffusion assay of CWG281 with pBAD24-*wza*$_{K30}$. Approximately 20 ml of LB-Amp with various concentrations of arabinose were plated to a measured depth of 6 mm ± 1 mm. Overnight cultures of CWG281+pBAD24-*wza*$_{K30}$, CWG281+pBAD24-GFP, CWG281, and K30 E69 were diluted down to an OD600 of 2.5 before being streaked onto their respective plates using a sterile swab. Using sterile forceps, 2 erythromycin disks were placed on top. Plates were incubated for 48 hours before checking for zones of inhibition (4).

Fluorescent microscopy GFP expression in CWG281 with pBAD24-GFP. Two 5 ml tubes of LB-Amp were inoculated with CWG281+pBAD24-GFP and CWG281+pBAD24-*wza*. The cultures were grown overnight with shaking at 37°C. L-arabinose was added to the CWG281+pBAD24-GFP culture to a final concentration of 1% and placed in an incubator for 20 minutes. Samples of each culture were placed onto slides with coverslips and viewed at 100x under UV light to confirm presence of GFP expression.

RESULTS

Preparation of pBAD24-*wza*$_{K30}$ construct and transformation into *E. coli* CWG281. In this study, the inducible pBAD24 expression vector was used to allow for tight regulation of *wza* expression from pBAD24-*wza*$_{K30}$. The pBAD vector contains the promoter of the arabinose operon (araBAD) as well as its regulatory gene *araC* (11). AraC is both a positive and negative regulator of the arabinose operon (11). In the presence of arabinose, AraC upregulates expression from the araBAD promoter, increasing transcription levels (11). In the absence of arabinose, AraC downregulates expression from the araBAD promoter and transcription occurs at low levels (11). Transcription can be further repressed through the addition of glucose (11). Glucose reduces levels of 3',5'-cyclic AMP, preventing the catabolite activator protein (CAP) from activating expression from araBAD (11).

To construct pBAD24-*wza*$_{K30}$, we first amplified *wza* from *E. coli* K30 E69 genomic DNA using primer pair Gibson forward and reverse (Table 1), containing flanking sequences homologous to pBAD24, necessary for Gibson assembly. Six base pairs upstream of the *wza* translational start site were included in the amplicon to optimally space the pBAD24 Shine-

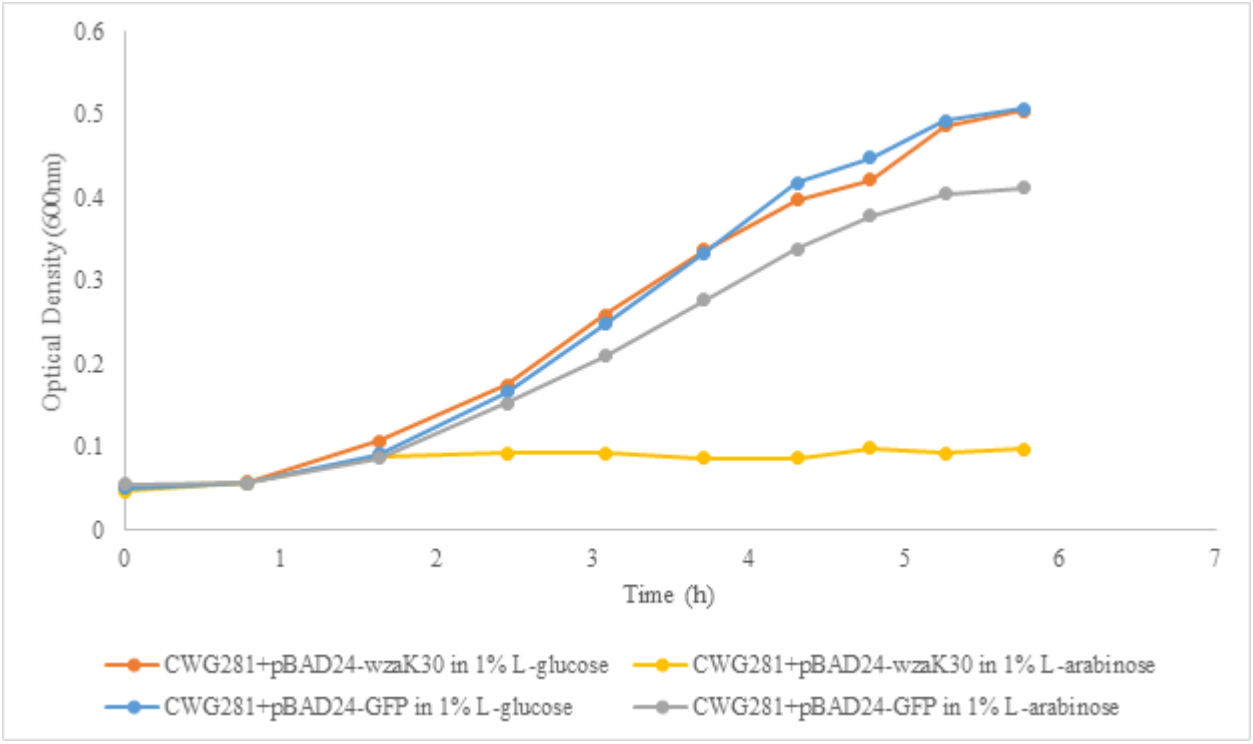


FIG. 4 Induction of pBAD24-*wza* in *E. coli* CWG281 with 1% L-Arabinose impairs growth. In 1% L-glucose, growth of *E. coli* CWG281+pBAD24-wza_{K30} and *E. coli* CWG281+pBAD24-GFP occurs at similar rates. In 1% L-arabinose, growth of *E. coli* CWG281+pBAD24-GFP occurs at a moderately reduced rate while growth of *E. coli* CWG281+pBAD24-wza_{K30} is substantially decreased.

Dalgarno sequence and the *wza* methionine start codon. The *wza* amplicon was cloned into the EcoRI/PstI-linearized pBAD24 vector using a commercially purchased Gibson assembly cloning kit and transformed into chemically competent *E. coli* DH5α cells. Seven transformants were obtained. Plasmid DNA isolated from three of the resulting transformants was screened by sequencing to detect the presence of the *wza* insert. One plasmid contained a wild-type version of *wza* (Figure 3); this pBAD24-wza_{K30} vector was subsequently transformed into *E. coli* CWG281. A commercially purchased pBAD24-GFP vector was also transformed into *E. coli* CWG281 to be used as a control in downstream growth curve analysis and Kirby-Bauer assays.

Overexpression of *wza* in *E. coli* CWG281+pBAD24-wza_{K30} is lethal. Lethality of *wza* overexpression was suggested by Yuen *et al.*, as a result we performed a growth curve analysis of CWG281+pBAD24-wza_{K30} using 1% L-arabinose and 1% L-glucose to assess whether overexpression of *wza* impaired cell growth (9). As a control, GFP was expressed in CWG281+pBAD24-GFP under the same conditions. In liquid media, 1% L-arabinose induction was shown to impair growth of CWG281+pBAD24-wza_{K30}. Additionally, in 1% L-arabinose, cell density reached a peak OD600 of only 0.1 after 6 hours while in 1% L-glucose a peak OD600 of 0.5 was reached (Figure 4). Growth of CWG281+pBAD24-GFP was only moderately reduced in 1% L-arabinose compared to 1% L-glucose (Figure 4). Taken together, overexpression of *wza* with 1% L-arabinose induction leads to decreased cell viability in *E. coli* CWG281+pBAD24-wza_{K30}.

To further characterize growth impairment, *wza* expression was induced in CWG281+pBAD24-wza_{K30} using various concentrations of L-arabinose (0%-0.010%), as well as 1% L-glucose (Figure 5). Growth occurred at the highest rate in 1% L-glucose and was further reduced in 0.001%-0.010% L-arabinose. Similar growth rates were observed at these L-arabinose concentrations (Figure 5).

Complementation of *wza* in *E. coli* CWG281 restores erythromycin sensitivity at 0.008% arabinose induction. To assess erythromycin-sensitivity in *E. coli* CWG281+pBAD24-wza_{K30}, Kirby-Bauer disk diffusion assays were conducted on LB agar supplemented with various concentrations of L-arabinose (0.001%-1%). As *wza* overexpression was previously established to result in decreased cell viability (Figure 4), optimal induction levels are needed to assess erythromycin sensitivity. If arabinose concentrations are too high, cell death as a result of *wza* overexpression cannot be distinguished from death occurring due to erythromycin-susceptibility. Conversely, if arabinose concentrations are too low, growth due to lack of *wza* expression cannot be distinguished from growth due to erythromycin-resistance.

Induction with 0.001%-0.010% L-arabinose was shown to modestly reduce CWG281+pBAD24-wza_{K30} growth by similar amounts, indicating that levels of *wza* expression across these arabinose concentrations are moderate and comparable (Figure 5). As such, the optimal induction level for restoration of erythromycin-sensitivity was thought to be between 0.001% and 0.010% L-arabinose. Furthermore, at higher concentrations of arabinose, increased erythromycin-sensitivity is expected as elevated Wza levels increase erythromycin import (7, 8). Thus, arabinose concentrations at the higher end of this range (0.006%-0.010%) were expected to restore erythromycin-sensitivity.

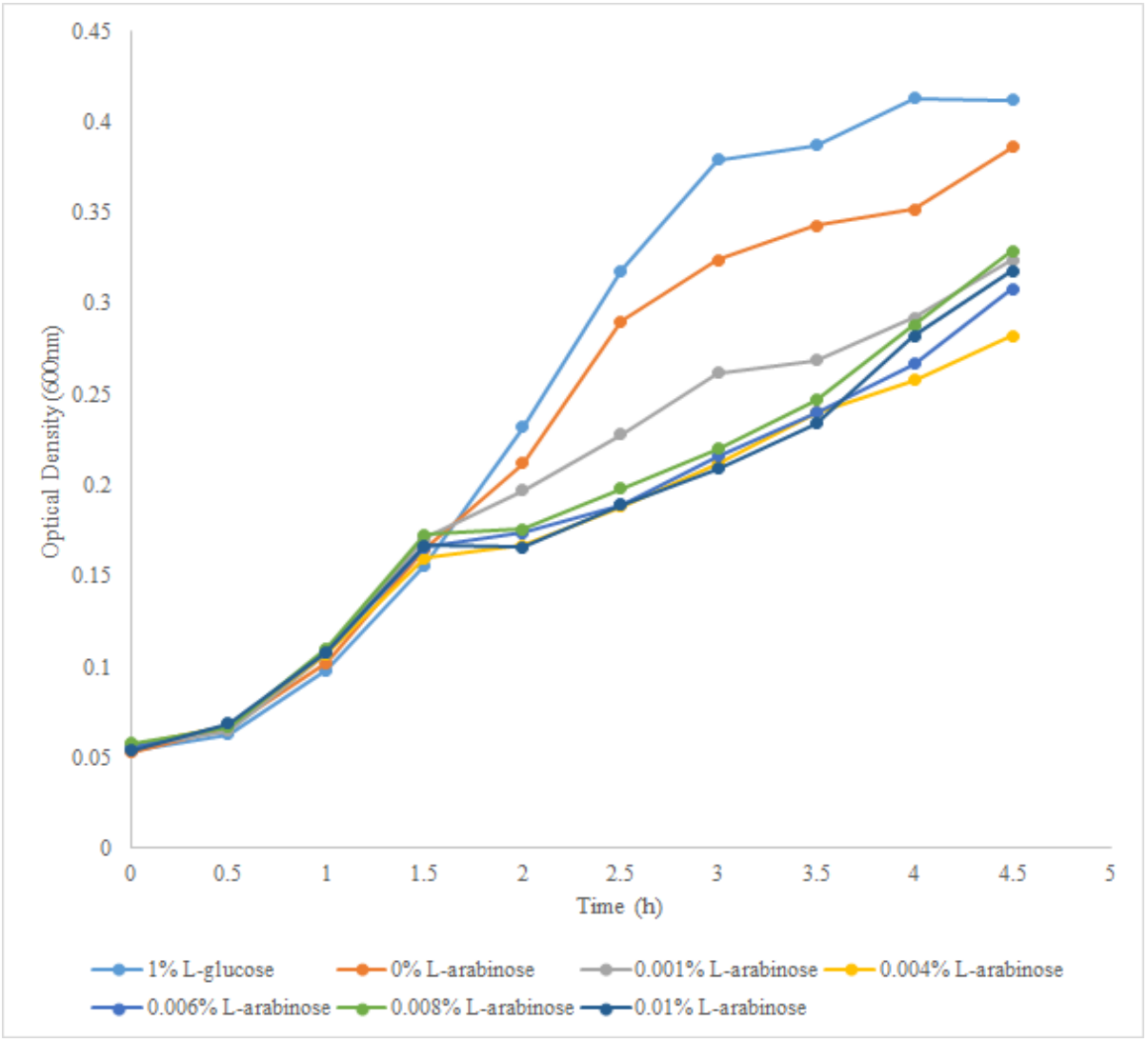
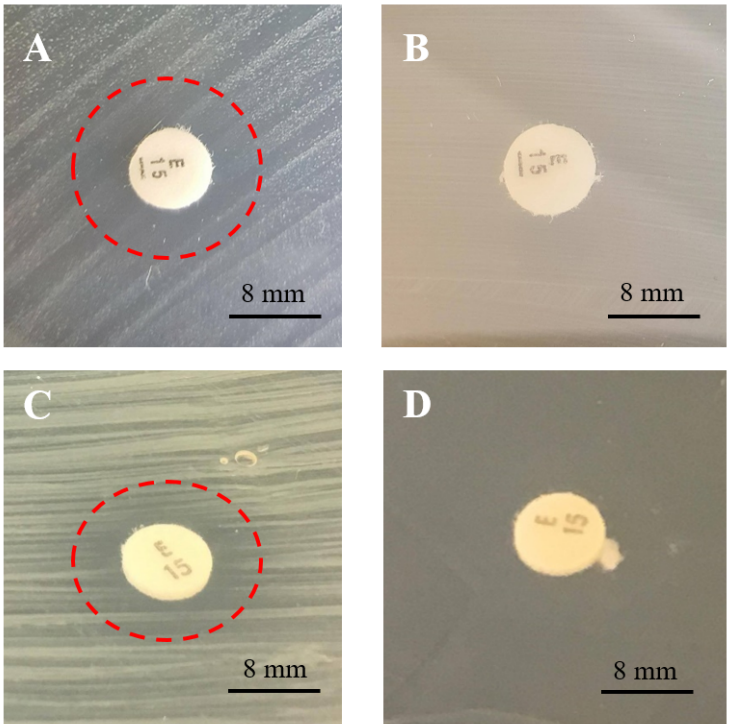


FIG. 5 Growth of *E. coli* CWG281+pBAD24-wza_{K30} at 37°C in liquid LB-Amp media supplemented with 1% L-glucose, 0% L-arabinose, 0.001% L-arabinose, 0.004% L-arabinose, 0.006% L-arabinose, 0.008% L-arabinose and 0.010% L-arabinose. Growth occurs at the highest rate in 1% L-glucose. Growth occurs at a decreased rate in 0% arabinose. Growth occurs at similar rates in 0.001%-0.010% arabinose.

To test these predictions, CWG281+pBAD24-wza_{K30} cells were first grown overnight in LB broth with 1% glucose, allowing for *wza* repression and optimal cell growth. Cultures were the diluted to an OD600 of 2.5 and immediately plated onto LB agar supplemented with L-arabinose. Plates were incubated for 48 hours before measuring zones of inhibition. As a control, erythromycin-sensitivity of *E. coli* CWG281+pBAD24-GFP was assessed using the same conditions.

At 0.008% arabinose induction, we found CWG281+pBAD24-wza_{K30} to be erythromycin-sensitive (Figure 6A) and CWG281+pBAD24-GFP to be erythromycin-resistant (Figure 6B). The complemented strain CWG281+pBAD24-wza_{K30} showed zones of inhibition 12 mm in diameter, similar in size to those seen in the wild-type K30 strain (Figure 6A, 6C). The CWG281+pBAD24-GFP control showed no zones of inhibition similar to the erythromycin-resistant phenotype of *E. coli* CWG281 (Figure 6B, 6D).



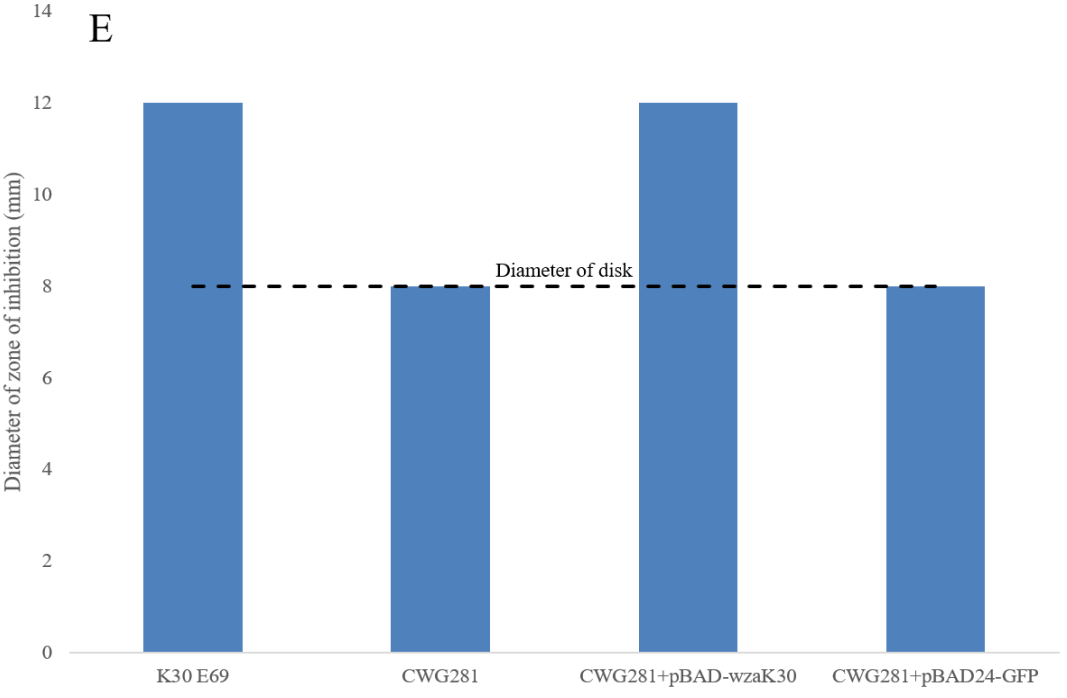


FIG. 6 Complementation of *wza* in *E. coli* CWG281 restores erythromycin sensitivity as determined by Kirby-Bauer disk diffusion assay. Disk diffusion assays were conducted on LB-Amp with 0.008% L-arabinose (A, B) or LB (C, D) agar using 15 μg erythromycin disks. Zones of inhibition are indicated with red dotted lines. *E. coli* CWG281+pBAD24-wza_{K30} (A) and *E. coli* K30 E69 (C) show zones of inhibition. *E. coli* CWG281+pBAD24-GFP (B) and *E. coli* CWG281 (D) show no zones of inhibition. (E) Diameter of zones of inhibition (mm) seen in (A-D) are shown graphically.

Erythromycin sensitivity of *E. coli* CWG281+pBAD24-*wza*$_{K30}$ is restored only at select L-arabinose induction levels. Kirby-Bauer assays conducted using high (1%, 0.1%) and low (0.001%) levels of arabinose induction showed no restoration of erythromycin sensitivity (Figure 7). 1% arabinose induction resulted in non-confluent growth of CWG281+pBAD24-*wza*$_{K30}$ colonies, likely due to *wza* overexpression (Figure 7). Interestingly, 0.1% arabinose induction resulted in confluent growth of CWG281+pBAD24-*wza*$_{K30}$; however, zones of inhibition were absent (Figure 7). At 0.001% arabinose, confluent growth was observed throughout without any zone of inhibition, likely attributed to insufficient levels of *wza* expression (Figure 7).

At 0.010% and 0.006% arabinose induction levels, partial restoration of erythromycin sensitivity was observed. Confluent growth occurred throughout with the exception of a zone of decreased growth density containing single isolated colonies surrounding the erythromycin disk (Figure 7). Although this zone cannot be deemed a true zone of inhibition, the reduction in growth density indicates partial restoration of erythromycin-sensitivity in CWG281+pBAD24-*wza*$_{K30}$ cells induced with these concentrations of arabinose.

DISCUSSION

In erythromycin-sensitive *E. coli* K30, Wza has been implicated in the transport of macrolides, such as erythromycin, across the OM (4, 7-9). Previous studies have proposed Wza to act as a channel, allowing erythromycin to bypass the OM permeability barrier and enter the periplasm (Figure 2); however, this model has not been fully tested (4, 7-9). To further characterize the role of Wza in *E. coli* K30 erythromycin-sensitivity, we attempted to complement *wza* in the *wza* deletion mutant *E. coli* CWG281. *wza* isolated from *E. coli* K30 was cloned into the arabinose inducible pBAD24 vector and transformed into *wza* deletion strain CWG281. Erythromycin susceptibility of the transformant was assessed using a

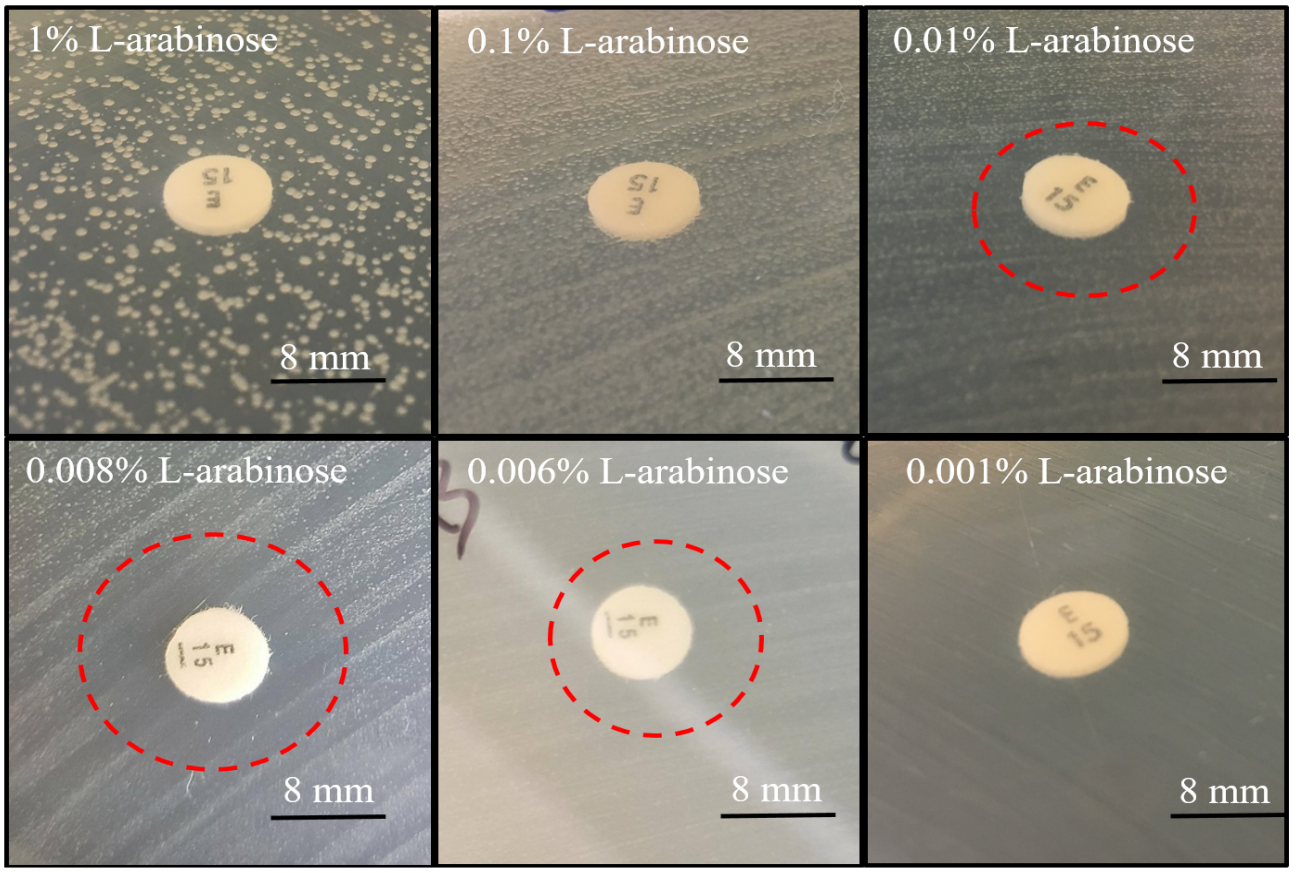


FIG. 7 Erythromycin sensitivity of *E. coli* CWG281+pBAD24-*wza*$_{K30}$ is restored only at select L-arabinose induction levels. Disk diffusion assays were conducted on LB-Amp agar with 0.001-1% L-arabinose using 15 μg erythromycin disks. Restoration of erythromycin sensitivity is not seen at high (1%, 0.1%) or low (0.001%) L-arabinose concentrations. Full restoration is seen at 0.008% L-arabinose while partial restoration is seen at 0.01% and 0.006% L-arabinose.

modified Kirby-Bauer disk diffusion assay. As seen in Figure 6, induction with 0.008% arabinose restored an erythromycin-sensitive phenotype in CWG281; however, induction using lower (e.g. 0.001%) or higher (e.g. 1%) concentrations of arabinose did not (Figure 7). Interestingly, growth curve analysis of CWG281+pBAD24-*wza*$_{K30}$ revealed substantially decreased growth rates in 1% arabinose in comparison to 1% glucose (Figure 4). The growth curve of CWG281+pBAD24-*wza*$_{K30}$ repressed with 1% glucose reached a peak OD600 of 0.5 after 6 hours, while CWG281+pBAD24-*wza*$_{K30}$ induced with 1% arabinose maintained a lower OD600 of 0.1 (Figure 4).

Erythromycin sensitivity of *E. coli* CWG281+pBAD24-*wza*$_{K30}$ is restored only at specific L-arabinose concentrations. Partial restoration of erythromycin-sensitivity was observed with the addition of 0.01% and 0.006 % L-arabinose to growth media (Figure 7). In these disk diffusion assays, zones of decreased growth density were observed surrounding erythromycin disks. These zones lacked confluent growth, and contained distinct, isolated colonies. We propose two explanations to account for the growth of colonies within these zones.

First, it is possible that mutations may arise in *wza* during plasmid replication, resulting in loss of Wza function. Mutated Wza may be unable to facilitate the transport of erythromycin across the OM, resulting in erythromycin-resistance. Selective pressure may also contribute to this process as overexpression of Wza was observed to be lethal (Figure 4). At sub-lethal arabinose induction levels, colonies carrying a mutated copy of *wza* may have a growth advantage. This selective pressure is likely more evident at higher arabinose concentrations and may explain why some colonies remained erythromycin-resistant at 0.01% arabinose.

Second, the araBAD promoter present in pBAD24 is known to show non-uniform induction at sub-saturating concentrations of arabinose (20-22). Arabinose induction operates in an autocatalytic mechanism, wherein intracellular arabinose levels can increase uptake of extracellular arabinose by upregulating expression of transporters (22). This leads to rapid accumulation of the inducer within cells (22). At any given time, stochastic processes allow for occasional expression of arabinose transporters, even in the absence of the inducer (22). The concentration of transporters will decrease as cells divide; however, if arabinose is introduced into the culture, cells expressing arabinose transporters will upregulate transporter expression and rapidly accumulate inducing levels of arabinose (22). As such, only a fraction of cells will achieve induction (20, 22). This fraction increases with increasing arabinose concentration and thus, at sub-saturating concentrations of arabinose, non-uniform induction of cells is observed (20, 22). This mechanism may explain why a subset of colonies remains erythromycin-resistant at low levels of arabinose (Figure 7). These colonies may result from the fraction of cells that do not achieve inducing levels of arabinose, and thus do not express sufficient levels of Wza necessary for restoration of erythromycin-sensitivity.

Interestingly, at 0.1% arabinose induction, erythromycin-sensitivity was not restored in *E. coli* CWG281. Moreover, growth was not observed to be impaired on solid media (Figure 7). We propose that increased expression of Wza results in overproduction of CPS, accounting for the observed erythromycin-resistance at 0.1% arabinose induction. Group 1 capsules have been shown to confer resistance to antimicrobial compounds by preventing these molecules from penetrating into the cell (1, 3). Although erythromycin-resistance in CWG281 is capsule-independent and capsule synthesis is not shown to confer resistance in K30, it is possible that overproduction of CPS may present a novel mode of resistance in *E. coli* CWG281. Wild-type CPS levels produced by K30 may not be sufficient to prevent erythromycin from penetrating the capsule barrier. It is possible that increased CPS production through increased Wza expression may be sufficient to inhibit antibiotic entry; increased CPS production has been previously shown to increase resistance to kanamycin and streptomycin in *E. coli* (1, 3). To test this hypothesis, a comparison must be made between CPS levels in *E. coli* K30 cells and CPS levels in CWG281+pBAD24-*wza*$_{K30}$ cells induced with 0.1% arabinose. Differential interference contrast (DIC) microscopy can be conducted using cells stained with India ink to determine whether capsules in CWG281+pBAD24-*wza*$_{K30}$ cells induced with 0.1% arabinose are larger than those in wild-type K30 cells. To confirm that growth of CWG281+pBAD24-*wza*$_{K30}$ is not impaired in 0.1% arabinose, growth curve analysis should also be conducted.

Overexpression of *wza* in *E. coli* CWG281+pBAD24-*wza*$_{K30}$ is lethal. Overexpression of *wza* through 1% arabinose induction was observed to substantially impair growth of CWG281+*wza*$_{K30}$ (Figure 4). We propose two mechanisms that may explain the observed growth impairment.

First, it is possible that Wza overexpression may result from saturation of the Sec translocon at the inner membrane. Outer membrane proteins (OMPs) are synthesized in the cytoplasm and trafficked to the inner membrane by the signal recognition particle (SRP) pathway (23, 26). Once at the inner membrane, OMPs are transported into the periplasm by the Sec translocon and subsequently inserted into the OM (23, 26). Upon overexpression of OMPs, Sec translocon capacity is exceeded, resulting in alteration of the cell envelope proteome as well as the aggregation of OMP precursors and secretory proteins in the cytoplasm (26). Alteration of the cell envelope proteome hinders cell division, thus resulting in decreased growth rates (26). Moreover, respiratory chain complexes at the inner membrane are reduced as a result of competition from OMPs for use of the Sec translocon, further reducing growth rates (26).

Observed growth impairment upon Wza overexpression may also result from insertion of large quantities of the protein into the OM. Classically, OMPs are assembled and inserted into the OM by the BAM and TAM complexes, which catalyze the insertion of beta strands in the OM (23). While most integral OMPs are characterized by beta-barrel topology, Wza is structurally nontraditional, with a transmembrane domain comprised solely of alpha-helices (23). Wza insertion into the OM is independent of the BAM and TAM complexes; studies have shown that when endogenous BAM and TAM expression are repressed, *wza* expression remains induced and Wza is detectable on the OM (23). An alternative insertion mechanism is proposed by Jeeves *et al.*, whereby Wza is inserted into the OM by the LOL machinery (23). The LOL machinery targets lipoproteins, such as Wza, to the OM, where homo-oligomerization of subunits occurs (23). During this homo-oligomerization event, it is believed that a conformational change occurs, driving the insertion of the C-terminal end of Wza into the membrane, forming a pore through the OM (23). Overexpression of *wza* may result in the formation of numerous pores within the OM, contributing to membrane instability and damaging the OM barrier. Loss of the OM permeability barrier results in increased passage of large molecules, such as antibiotics, through the cell envelope, resulting in toxicity (24, 25). In this study, damage to the OM barrier in CWG281+pBAD24-*wza*$_{K30}$ may result in increased entry of ampicillin into the cell. Although CWG281+pBAD24-*wza*$_{K30}$ carries the beta-lactamase gene (*amp*^{R}) on the pBAD24-*wza*$_{K30}$ vector, levels of intracellular ampicillin may saturate the enzymatic capacity of expressed beta-lactamases. As a result, excess amounts of uncleaved ampicillin may overwhelm the bacteria, impairing growth of cells overexpressing Wza.

Limitations The main challenge in this study was the optimization of L-arabinose concentrations for pBAD24-*wza*$_{K30}$ induction that would allow for the restoration of erythromycin-sensitivity without impairing bacterial growth. Additional disk diffusion experiments conducted at the optimal arabinose inducer concentration (0.008%) would increase the statistical robustness of our findings.

Conclusions In conclusion, we found that induction of *E. coli* CWG281+pBAD24-*wza*$_{K30}$ with 0.008% L-arabinose restores erythromycin-sensitivity and that overexpression of Wza using higher concentrations of L-arabinose (e.g. 1%) impairs growth. This study suggests that complementation of *wza* in *E. coli* CWG281 is sufficient to restore an erythromycin-sensitive phenotype supporting a working sensitivity model involving Wza.

Future Directions In erythromycin-sensitive *E. coli* K30, single-deletions of *wza* and *wzc* give rise to erythromycin-resistant strains, suggesting that both Wza and Wzc are necessary for erythromycin import into the cell (7, 8). However, it is unclear whether both proteins are necessary for import of erythromycin into the periplasm. In CPS export, Wzc interacts with Wza to induce a conformational change necessary for forming the Wza channel at the periplasmic face (13). This allows for export of CPS, and likely import of macrolides;

however, experimental data has not been generated to support this hypothesis. An alternative mechanism may involve Wza specifically translocating erythromycin into the periplasm and Wzc importing the antibiotic into the cytoplasm (8). Future studies may wish to observe the localization of erythromycin in the *wzc* knockout strain CWG285. If erythromycin localizes outside of the cell in CWG285, it is likely that both Wza and Wzc are necessary for erythromycin import into the periplasm. If erythromycin localizes within the periplasm in CWG285, it is likely that only Wza is necessary for transport of erythromycin into the periplasm. Preliminary studies describe the potential of a fluorimetric method to assess erythromycin localization in the cell, which may be utilized to determine erythromycin localization in *E. coli* CWG285 (27).

Future studies may also address whether sensitivity to other macrolides is restored by complementation of *wza* in *E. coli* CWG281. Botros *et al*. observed deletion of *wza-wzb-wzc* to confer resistance to not only erythromycin but clarithromycin and roxithromycin; therefore, it is possible that complementation of *wza* in *E. coli* CWG281 also restores sensitivity to clarithromycin and roxithromycin (4).

In this study, we observed induction of CWG281+pBAD24-wza_{K30} with 0.08% arabinose to be sufficient to restore erythromycin-sensitivity while induction with 0.1% arabinose was found to be insufficient (Figure 7). Increasing arabinose levels should increase Wza production, which is expected to increase erythromycin sensitivity; however, this was not observed. We propose that the erythromycin resistance in CWG281+pBAD24-wza_{K30} following 0.1% arabinose induction is due to increased expression of CPS, which confers capsule-dependent erythromycin-resistance to CWG281. Future studies may attempt to analyze whether increased CPS production is observed in CWG281+pBAD24-wza_{K30} following 0.1% arabinose by comparing CPS levels at various arabinose concentrations. DIC microscopy and India ink staining may be used to visualize capsules.

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CONTRIBUTIONS

AP: Wrote the introduction and results sections, edited the methods and material section, wrote portions of the discussion, and future directions sections, and generated Figure 1-6, S1-S3. **MR**: Wrote material and method, discussion, and portions of limitations, references, and editing. **PT**: Wrote the abstract, portions of the methods, discussion section, future directions, and limitations, generated Figure 4 and edited the introduction. **JW**: Wrote material and method section, conclusion section, acknowledgement section, portions of reference section and portions of future direction section.

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