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Investigation of Wza in Erythromycin Sensitivity of Escherichia coli K30 E69 by Genetic Complementation

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***Escherichia coli* K30 strains express group 1 capsule that is polymerized and transported by the Wzy-dependent pathway. This involves proteins encoded by genes *wza, wzb,* and *wzc*. Previous studies have found that deletions of *wza* and *wzc* from the erythromycin sensitive K30 wild-type strain results in a change to an erythromycin resistant phenotype. It is unknown why the deletions of *wza* and *wzc* confers resistance to erythromycin. In this study, we attempted to complement a *wza* deletion mutant with a plasmid encoded copy of *wza*. We first reproduced the previously reported erythromycin sensitive phenotype of the wild-type K30 strain E69 and the resistant phenotype of the *wza* mutant strain CWG281 on plates. We then constructed a plasmid encoding the *wza* coding sequence with a 200 base pair putative regulatory region upstream of the methionine translational start site. Complementation of CWG281 with our *wza* plasmid construct did not result in reversion to an erythromycin sensitive phenotype. Further investigation to evaluate Wza protein expression level in the *wza* complemented strain is required to validate the requirement of Wza in erythromycin sensitivity.**

Macrolides are a class of antibiotics that inhibit bacterial protein synthesis by binding the 23S ribosomal RNA of the 50S ribosomal subunit (1). These hydrophobic antibiotics access the bacterial cytoplasm by diffusing through the lipid membrane bilayer. Most Gram-negative bacteria such as *Escherichia coli* (*E. coli*) are resistant to macrolides due to the presence of their outer membrane (OM). Lipopolysaccharides (LPS) and proteins in the asymmetric outer leaflet of the OM are a barrier to diffusion of hydrophobic antibiotics into the cell (2). However, studies on *E. coli* K30 E69 strain show that it is sensitive to the macrolide erythromycin in Kirby Bauer disc diffusion assays of antibiotic sensitivity (3, 4).

One of the extracellular and surface virulence factors that are found in the Gram-negative bacteria *E. coli* is the capsule. Capsules surround the outermost layer of the cell and are composed of polysaccharide chains known as capsular polysaccharides (CPS) (5). The CPS layer can be typically up to 400 nm thick and 200 sugars long (6). CPS are classified into four groups based on the organisation of capsule gene clusters, the assembly pathway and regulatory features of capsule expression (7). *E. coli* K30 E69 produce a group 1 capsule, which is assembled using the Wzx, Wzy, Wza, Wzb, and Wzc proteins (4, 7) (Fig. 1). The latter 3 proteins are produced from the Wzy cassette (3). Wzx is a flippase, which flips a polysaccharide precursor into the periplasm between the inner and outer membrane (8). Wzy is an O-antigen polymerase which extends the polysaccharide chain (9). Wza is an OM lipoprotein which exports the CPS from the periplasm to the OM surface (10).

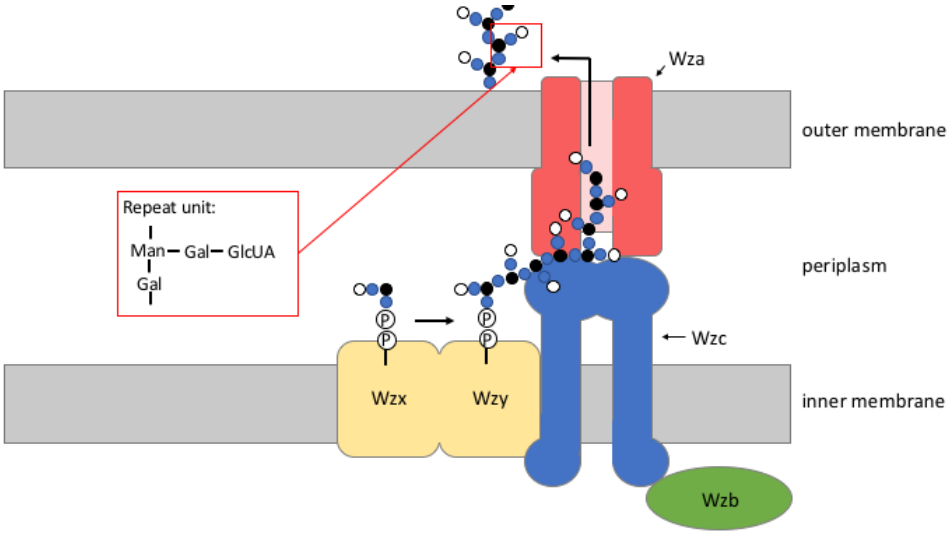

FIG. 1 Cartoon cross section of the proteins involved in the Wzy-dependent pathway of capsule production. Each protein is labelled with its name by direct labelling or with arrows. The gray layers indicate the membrane bilayers of *E. coli* K30 E69 (WT). Wza and Wzc form a tight complex which translocates the polysaccharides which form the capsule, working in conjunction with Wzx and Wzy in the production of group 1 capsule. Wzb performs phosphatase activity on Wzc. The polysaccharide chain consists of repeating units of mannose (Man), galactose (Gal), and glucuronic acid (GlcAU).

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Wzc is an inner membrane autokinase with Wzb as its cognate phosphatase (11). Wzc phosphorylation is key in regulating the synthesis and export of CPS (12).

Knockout mutants of members of the Wzy-dependent pathway in *E. coli* K30 E69 have shown resistance to erythromycin (4, 11, 13). Botros *et al*. found that deletion of *wza*, *wzb*, and *wzc* together confers resistance to erythromycin independent of capsule formation (4). Su *et al*. and Jazdarehee *et al*. have found, respectively, that single deletions of *wza* and *wzc* confer resistance to erythromycin in solid media (13, 11), while Rana *et al*. found that *wzb* deletion does not confer resistance (3). Su *et al*. and Jazdarehee *et al*. have hypothesized a possible mechanism of Wza acting as a pore and allowing increased influx of erythromycin into the cytoplasm (13, 11). Conversely, Delcour notes that hydrophilic antibiotics use porins to diffuse into cells (2). Hydrophobicity can be defined by the octanol-water partition coefficient, a ratio where values greater than one mean the solute partitions more preferentially into octanol than water. Only drugs with an octanol-water partition coefficient less than 0.1 seem to enter through porins (14), however, erythromycin has an octanol-water coefficient of 1.6 (15). Therefore, it seems unlikely that erythromycin directly uses Wza as a pore. It has been shown that Wza and Wzc form a highly specific complex, which brings the outer and the inner membrane together locally, resulting in a periplasmic space that is locally 105 to 125 angstroms thick (12). As deletion of either Wza or Wzc alone is enough to confer resistance (4,13), it is possible that the interaction between Wza and Wzc may be key to erythromycin resistance. However, the exact mechanism is yet to be elucidated.

In order to investigate the dependence of Wza in erythromycin sensitivity, we decided to perform a *wza* complementation study using a *wza* deletion mutant strain, CWG281 (10). Complementation of the *E. coli* strain CWG281 (*wza*$_{22\ min}$::*aadAwza*$_{K30}$::*aacC1*) with Wza from *E. coli* K30 E69 (WT) on an arabinose-induced pBAD vector has restored *E. coli* K30 CPS synthesis and surface expression (10), so it is possible that erythromycin sensitivity could also be restored. In our experiment, we have attempted to use TOPO® as an alternative vector for transformation as it is simple, cost and time efficient. If complementation is successful it demonstrates a proof of concept that TOPO® vectors can be used for complementation of membrane proteins.

We hypothesized that complementation of CWG281 with *wza* would be able to restore erythromycin sensitivity. In this study, we used a TOPO® plasmid containing the *wza* gene and its promoter to complement CWG281. The *wza* TOPO® plasmid construct did not restore an erythromycin sensitive phenotype. Our result can not exclude the possibility that the retained erythromycin resistant phenotype is due to a technical issue with the complementation system. Examination of Wza protein expression in this complemented CWG281 (CWG281^{C}) is required for an unbiased conclusion.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* K30 E69 (WT) (serotype: O9a:K30:H12) and CWG281 (*wza*$_{22\ min}$::*aadAwza*$_{K30}$::*aacC1*) strains were obtained from the laboratory of Dr. Chris Whitfield from the University of Guelph's Department of Molecular and Cellular Biology. *E. coli* K30 E69 is the isogenic parental strain of CWG281. Bacterial strains were isolated for use on Lysogeny broth (LB) agar plates (1.0% w/v NaCl, 1.0% w/v Tryptone, 0.5 % w/v yeast extract, 2.0% w/v agar), and were grown in both LB broth (1.0% w/v NaCl, 1.0% w/v Tryptone, 0.5 % w/v yeast extract) or Mueller-Hinton (MH) broth (0.3% w/v beef extract, 1.75% w/v casamino acid, 0.15% w/v starch).

Oligonucleotide primer design. The oligonucleotide primers for the polymerase chain reaction (PCR) amplification used in this study were designed using the *E. coli* K30 E69 (accession no. AF104912.3). In an attempt to include the native promoter, the oligonucleotide primers were designed 221 base pairs (bp) upstream (in attempt to include the native promoter) and 44 bp downstream to the *E. coli* K30 E69 *wza* gene. The forward and reverse oligonucleotide primers were designed using the software from Primer3 (16). Both primers were determined to have a similar GC content of 50% and a similar melting temperature (Tm) using the Tm calculator from Integrated DNA Technologies (IDT) website (Table 1).

PCR and agarose gel electrophoresis. Primers used to amplify the *wza* region were purchased from IDT. The primers were resuspended to a final concentration of 100 µM using sterile distilled water as a solution stock. *E. coli* K30 E69 genomic template DNA was extracted by the PureLink® Genomic DNA kit. PCR amplification was performed with the Invitrogen™ Platinum™ *Taq* DNA Polymerase. Each 50 µL PCR reaction contains: 5 µL of 10X PCR Buffer, 1.5 µL of 50 mM $MgCl_2$, 1 µL of 10 mM DNTPs, 1 µL of 10 mM forward primer, 1 µL of 10 mM reverse primer, 1.85 µL of *E. coli* K30 E69 genomic DNA, 0.2 µL of Platinum™ *Taq* DNA polymerase, and 38.45 µL of sterile distilled water. The PCR amplification conditions were 94°C for 120 seconds for the initial denaturation, followed by 30 cycles of 94°C for 30 seconds, 51.8°C for 30 seconds, and 72°C for 60 seconds with a final extension at 72°C for 10 minutes. The PCR product was examined on a 2% (w/v) agarose gel containing SYBER safe DNA dye (ThermoFisher Scientific). A 2.5 µL sample of PCR product was loaded with 0.5 µL of 6X DNA Gel loading dye. The agarose gel was run at 120 V for 45 minutes. The agarose gel was visualized using the Alpha Innotech Alphaimager.

Cloning of pCR®2.1-TOPO®-K30*wza* plasmid. The TOPO® plasmid was chosen as the vector for the simplest complementation experiment due to the availability of resources and the time constraints. The *wza* PCR product was cloned into the pCR®2.1-TOPO® vector using the TOPO® TA Cloning® Kit to form the recombinant plasmid we will call pCR®2.1-TOPO®-K30*wza*. One Shot® TOP10 competent *E. coli* cells were transformed with pCR®2.1-TOPO®-K30*wza* using the TOPO® TA Cloning® Kit. 50 µL of the transformation reaction was spread on 50 µg/mL LB-Kanamycin plates supplemented with 40 µL of 40 mg/mL X-gal. Spread plates were incubated at 37°C overnight for the growth of colonies. Cells containing pCR®2.1-TOPO®-K30*wza* have the alpha portion of the *lacZ* on the plasmid interrupted by *wza*, and the transformed cells will be selected for as a white colony on the X-gal plate.

DNA sequencing of pCR®2.1-TOPO®-K30*wza*. White colonies were picked from the X-gal plate of our cloning process. pCR®2.1-TOPO®-K30*wza* from these colonies were purified for sequencing. In order to verify the correct orientation of the insert and ensure the insert was free of PCR-induced errors, each sample was sequenced in the forward direction and reverse direction by the

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use of the universal primers M13 forward and M13 reverse respectively. Sequencing results were analyzed using SnapGene® software.

Genomic complementation. Genomic complementation was performed using chemically competent CWG281 transformed with pCR®2.1-TOPO®-K30*wza*. Competent CWG281 was prepared using a modified protocol indicated by Chung *et al*. (17). Briefly, a single colony of CWG281 was picked from an agar plate. A 5 mL overnight cell culture was prepared at 37°C. The culture was then diluted 1/100 in 5 mL final volume in fresh LB and grown until reaching 0.25-0.5 OD_{600}. The cells were resuspended and aliquoted in 10% of the original culture volume (0.5 mL) using TSS buffer. TSS was made using 10% (wt/v) PEG, 0.03 M $MgCl_2$ and 0.05% (v/v) DMSO diluted in LB medium. The competent cells were stored at -80°C in 100 µL aliquots.

The transformation was done by first thawing the 100 µL aliquots of competent cells on ice. After thawing, 1 µg of pCR®2.1-TOPO®-K30*wza* plasmid was added and incubated on ice for 30 minutes. The mixture was then incubated in 42°C water bath for 1 minute then transferred to ice. 1 mL of LB is then added to the mixture and incubated at 37°C for 1 hour. 50-150 µL of the mixture was plated on LB plates containing kanamycin (50 µg/mL) and ampicillin (50 µg/mL).

Disc diffusion assay. A modified version of the Kirby-Bauer disc diffusion susceptibility test used by Su *et al.* was used to investigate erythromycin sensitivity (13). Isolated colonies were taken from the respective stock plates and were grown in 2 mL of either LB broth or MH broth overnight at 37°C. An aliquot of the overnight culture was diluted to an OD_{600} of 1.0 in a final volume of 1 mL. 100 µL of the 1.0 OD_{600} aliquot was spread plated to the corresponding LB agar plates (1.0% w/v NaCl, 1.0% w/v Tryptone, 0.5 % w/v yeast extract, 2.0% w/v agar), or MH agar plates (0.3% w/v beef extract, 1.75% w/v casamino acid, 0.15% w/v starch, 2.0% w/v agar). Prior to the application of the erythromycin discs, the plates were left in a laminar flow cabinet with the lid half-off for 10 minutes to allow the drying of excess moisture. Forceps were sterilized with 70% ethanol and used to place 1 erythromycin disc to each half of the plate. The erythromycin discs were provided by the Department of Microbiology at UBC. After 20 hours, the plates were observed for the appearance of any inhibition zone (clearing in the bacterial lawn) around the erythromycin disc.

RESULTS

Confirmation of the erythromycin sensitivity phenotype of *E. coli* K30. Su *et al.* confirmed the erythromycin sensitivity phenotype of *E. coli* K30 E69 (sensitive) and CWG281 (resistant) using a disc diffusion assay (13, 4). To validate the phenotype and confirm the strain identities, we reproduced these previous experiments on both LB agar (Fig. 2A) and MH agar (Fig. 2B). Inhibition zones were observed around the erythromycin discs on the plates with the WT strain (Fig. 2Ai, 2Bi), suggesting *E. coli* K30 E69 is sensitive to erythromycin. No inhibition zones were seen on the plates with CWG281 (Fig. 2Aii, 2Bii) suggesting CWG281 is resistant to erythromycin. Our results confirmed the reported phenotype of our strains of interest.

Preparation of pCR®2.1-TOPO®-K30*wza*. In order to attempt a complementation of *wza* into CWG281, we used the high copy number TOPO® plasmid as the expression vector because of ease of availability and time constraints. We designed our complementation plasmid with *wza* and 200 bp upstream of the methionine translational start site,

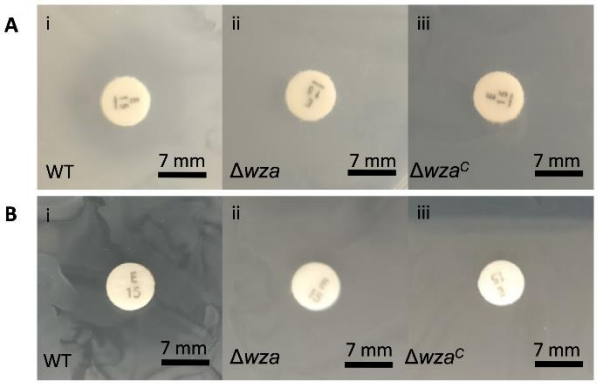

FIG. 2 Representative images from disc diffusion of WT, CWG281 and CWG281^C A represents disc diffusion assay done on Lysogeny broth agar, while B represents Mueller-Hinton agar. i, ii, and iii are respectively *E. coli* K30 E69 (WT), CWG281 (Δ*wza*) and CWG281^C (Δ*wza*^{C}). A clear inhibition zone can be seen with i but not with ii or iii on both types of agar.

in order include the native promoter to drive the expression of *wza*.

The PCR result showed a 1405 bp band on an agarose gel (Fig. 3). No band was observed in the PCR reaction without template DNA included in lane 2 as expected of a negative control. This suggested we successfully amplified the promoter-containing *wza* gene from *E. coli* K30 E69 genomic DNA. The PCR product was later cloned into the TOPO® vector, and the resultant vector was transformed into One Shot® TOP10 competent *E. coli*. Colonies which contained the recombinant plasmid, pCR®2.1-TOPO®-K30*wza*, were selected from blue-white screening. White colonies were expected to carry pCR®2.1-TOPO®-K30*wza* (Fig. 4).

We validated pCR®2.1-TOPO®-K30*wza* using sequencing, in order to determine in what orientation the PCR product had been inserted into the plasmid and that the *wza* gene in the inserted PCR product did not have

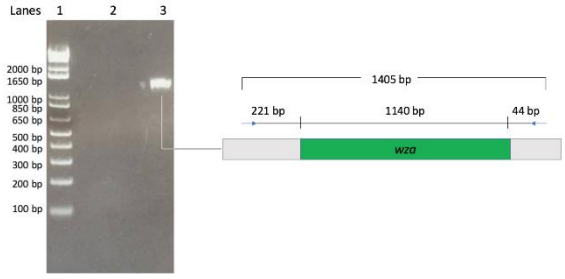

FIG. 3 PCR amplification of the *wza* gene in *E. coli* K30 E69 (WT). We designed forward and reverse primers (blue arrows) to amplify the *wza* gene of the capsule biosynthesis cluster of WT. The amplified region is predicted to be 1405 bp long. The *wza* gene is 1140 bp long according to the *E. coli* K30 capsule biosynthesis cluster, partial sequence, accession no. AF104912.3 from GenBank. The design of the PCR amplification includes 44 bp downstream of the *wza* gene and 221 bp upstream of the gene, which is where the endogenous promoter is suspected to reside. Lane 1: 1 Kb Plus DNA Ladder. Lane 2: Negative control with no WT template added. Lane 3: WT genomic template amplified with the designed primers.

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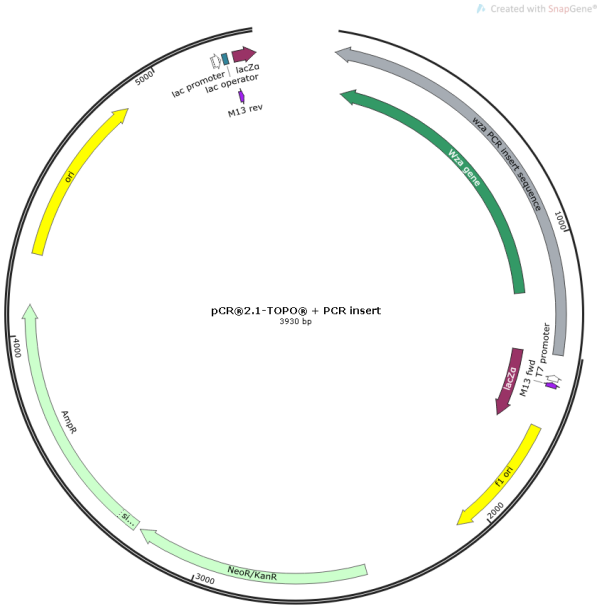

FIG. 4 Graphic representation of pCR®2.1-TOPO®-K30*wza*. The pCR®2.1-TOPO® + PCR insert map was created with the SnapGene® software. The PCR product that is inserted into the plasmid can be seen in gray, The *wza* gene is shown in dark green. The various other arrows indicate the features of the vector. The yellow arrows indicate the origins of replication (*ori*). The light green arrows indicate the ampicillin resistance gene and neomycin/kanamycin resistance genes respectively. The white arrows indicate both the *lac* promoter and T7 promoter. The turquoise arrow indicates the *lac* operator. The two burgundy coloured arrows indicate the *lac*Zα gene disrupted by the *wza* PCR insert sequence. The two purple arrows indicate the M13 reverse primer and the M13 forward primer.

mutations that would affect the function of the Wza protein. Of the 8 samples we sent for sequencing, one sample, which we designated as plasmid B**,** had an insert with 100% identity to the reference sequence *E. coli* K30 capsule biosynthesis cluster (Fig. S1). We decided to use plasmid B for complementation of CWG281. We expected *wza* to be expressed via the native promoter we attempted to clone into the insert.

Complementation with *wza* did not restore the erythromycin sensitive phenotype. CWG281 was transformed with pCR®2.1-TOPO®-K30*wza* and was found to remain resistant to erythromycin. No inhibition zone was observed on both LB agar (Fig. 2Aiii) and MH agar (Fig. 2Biii). CWG281 and the transformed strain, CWG281^{C}, appeared equally resistant to each other on both LB agar (Fig. 2Aii, 2Aiii) and MH agar (Fig. 2Bii, 2Biii).

DISCUSSION

In this study, we investigated the requirement of Wza in erythromycin sensitivity We hypothesized that complementation of CWG281 with *wza* would be able to restore erythromycin sensitivity. We first reproduced the previously reported erythromycin sensitive phenotype of *E. coli* K30 and the resistant phenotype of CWG281 on plates. We then constructed a plasmid encoding the *wza* coding sequence with a 200 base pair putative regulatory region upstream of the methionine translational start site.

In order to confirm the relationship between erythromycin sensitivity and the presence of *wza* gene expression, a complementation experiment was completed to determine whether the *wza* gene can restore erythromycin sensitivity in CWG281. Our result displayed that erythromycin resistance remained in CWG281^{C}. It is possible that our method of complementation was unsuccessful due to a lack of expression of the Wza protein in CWG281^{C}. We propose that the following explanations may account for the lack of observed complementation:

(i) No native promoter in the PCR generated insert
(ii) Antisense inhibition of the *wza* promoter
(iii) Cell toxicity selecting for an insert orientation that results in (i) or (ii)

Our insert is oriented as illustrated in Figure 4, with a T7 promoter upstream of the insert. However, the K30 *E. coli* would need to express T7 RNA polymerase to make use of this promoter (18). Instead, it's likely that in order for the gene to be expressed, it would require the native promoter to drive expression. Since we did not observe any change in erythromycin sensitivity after complementation, one possibility is that the PCR product, which included about 200 bp upstream of the *E. coli* K30 E69 *wza* gene, did not capture the native promoter. It has been suggested that the promoter of the capsule biosynthesis gene cluster of *E. coli* K30 is located about 1900 bp upstream of the *wza* gene (19) making it likely we have not included it in our insert.

Another possibility is that the downstream antisense *lac* promoter is inhibiting the expression of *wza*. A study using a synthetic antisense transcription system in *E. coli* has found that an antisense promoter can repress gene expression in proportion to the strength of the promoter (20). The constitutive strength of the *lac* promoter in our plasmid construct may be repressing gene expression. A combination of these reasons may lead to low or no expression of the Wza protein and prevent the restoration of erythromycin sensitivity in CWG281^{C}.

We expected that the PCR product insert would insert into the TOPO® vector in both possible orientations with the same probability as there is no preference in the TOPO® cloning reaction for one orientation over another because both strands of the insert have a 3' thymine overhang. To our surprise, all 8 samples that we analyzed indicated that they inserted in the same orientation which starts at the M13 forward primer as indicated in Figure 4. We hypothesize that there is selection for that orientation due to toxicity caused by overexpression of *wza* by the strong *lac* promoter in the opposite orientation.

The pCR®2.1-TOPO® vector that was utilized in this experiment uses a pUC ori, which is normally used to

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maintain a high copy number of the plasmid. The high copy number could result in overexpression of *wza* if the direction of expression of the gene in the insert was aligned with and downstream of the strong *lac* promoter on the plasmid (the orientation opposite to the one we observed). One Shot® TOP10 cells do not require IPTG so the *lac* promoter would be active to overexpress *wza* in these cells. The results of overexpressing membrane proteins are known to cause *E. coli* cell toxicity or form inclusion bodies (21). Alternatively, overexpression of Wza could cause the insertion of excess outer membrane pores leading to over-permeability of the outer membrane in the One Shot® TOP10 competent cells used in the TOPO® Cloning reaction kit. In *Saccharomyces cerevisiae*, a species of yeast, overexpression of the outer mitochondrial membrane protein channel, VDAC1, led to outer membrane permeability and induced apoptosis due to proapoptotic proteins leaking into the cytosol (22). If an analogous sequence of steps severely decreased the cell viability of transformants with the insert in this detrimental orientation then the plasmid constructs that were selected from the blue-white screening would only be from the viable cells with the insert in the orientation we observed.

Our results confirmed that deletion of the *wza* gene in E. coli K30 E69 results in resistance to erythromycin. However, further study is needed to determine whether *wza* is specifically responsible for erythromycin resistance. Our preliminary result suggests that Wza may not be solely responsible for erythromycin entry.

FUTURE DIRECTIONS

Examination of wza expression levels in CWG281^{C}. Due to the uncertainty of the expression levels of Wza in CWG281^{C}, a follow-up experiment should be done to evaluate the *wza* mRNA levels via Reverse Transcription-quantitative PCR (RT-qPCR). To have a better understanding of our current results, it would be key to observe whether the *wza* is actually transcribed from pCR®2.1-TOPO®-K30*wza*. Based on the observed *wza* mRNA levels from the RT-qPCR, it may be possible to understand which of the possible issues regarding pCR®2.1-TOPO®-K30*wza* that we have discussed is responsible for the lack of phenotypic complementation. If the Cq value is comparable to the WT and therefore significantly higher than CWG281, *wza* complementation at the mRNA level was successful at an appropriate dosage. Too high or too low dosage may indicate that the complementation is not physiologically relevant. As the primers for this experiment have already been designed, ordered, and received (Table S1), it would be a possible and useful experiment for a future team to complete as part of a follow-up to our project.

An associated experiment to RT-qPCR would be to measure the relative protein levels of Wza in CWG281^{C} compared to the WT. A direct approach such as Western Blot was not appropriate for us due to the low availability of anti-Wza antibodies and the inconsistent level of reliability of antibodies to uncommon antigens. A more appropriate and physiologically relevant approach would be to measure Wza expression by measuring the levels of capsule formation as Reid *et al.* have done (10). Botros *et al.* have also previously measured relative capsule formation by capsule staining in *E. coli* K30 E69, and have observed temperature dependent differences in capsule formation levels between the WT and the total Wzy cassette knockout strain (4). We expect the amount of capsule to be lower for CWG281 than the WT. If CWG281^{C} shows a recovery in capsule level towards the WT amount, then this would signify that the *wza* on our plasmid is being expressed.

Other complementation systems. In order to establish a more efficient complementation system, a method using a T7 RNA polymerase/promoter system may be considered (23). Poor expression of *wza* may be due to the lack of the native *wza* promoter in the insert, so we could transform a second plasmid containing a heat inducible T7 RNA polymerase to drive expression of *wza* using the T7 promoter upstream of the insert on the plasmid. The new complementation system will clarify our concerns on the efficiency of wza transcription from recombinant plasmid.

An alternative complementation technique is to integrate *wza* into the chromosome. Usage of Tn7 transposons have previously been demonstrated as a method to complement with physiologically relevant single gene dosage with good efficiency in *Enterobacteriaceae* such as *E. coli* (24), and could be applied to complementation of *wza*.

Induction of *wza* gene expression with an arabinose-induced pBAD vector could be used as another complementation approach. This system regulates and modulates gene expression more tightly than the TOPO® vector (25). The pBAD vector has previously been used by Reid *et al.* to restore K30 CPS synthesis in *E. coli* strain CWG281 using *wza* complementation (10). It would be interesting to explore whether erythromycin sensitivity can also be restored using the same system.

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