Undergraduate Journal of Experimental Microbiology and Immunology (UJEMI) Vol. 25:1-9


# Biofilm production in *Escherichia coli* K30 with group 1 capsular gene *wza* and *wza-wzb-wzc* deletions is not correlated with Erythromycin resistance phenotypes in liquid media

Cynthia James, Christine Kim, Catherine Pan, Douglas Zhong

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

**SUMMARY** The Wzy-dependent capsular polysaccharide export system consists of an outer membrane channel (Wza), inner membrane channel and tyrosine autokinase (Wzc), and regulatory phosphatase (Wzb), and is responsible for the export and assembly of extracellular polysaccharide capsules. In *Escherichia coli* K30, this system is critical for the formation of group 1 capsules. Previous studies have shown that *E. coli* K30 Δ*wza* and Δ*wza-wzb-wzc* have greater resistance to erythromycin, a macrolide antibiotic, on solid media compared to wild-type K30; however, the opposite phenotype was observed in liquid media. Therefore, we hypothesized that the different erythromycin resistance phenotypes in *E. coli* K30 wild-type, Δ*wza*, and Δ*wza-wzb-wzc* are due to differences in biofilm production in solid and liquid media. In this study, we investigated whether biofilm formation is correlated with the differential erythromycin resistance phenotype of wild-type *E. coli* K30, Δ*wza*, and Δ*wza-wzb-wzc* in solid and liquid media. Erythromycin disc diffusion assays on solid media, minimum inhibitory concentration assays in liquid media and crystal violet biofilm assays were performed on all three strains. We found that Δ*wza* and Δ*wza-wzb-wzc* were more resistant than the wild-type strain on solid media, but all three strains had similar levels of resistance in liquid media. In contrast to previous studies, we obtained consistent erythromycin MIC values in liquid media and determined that it was between 125 and 250 μg/mL, but we were unable to obtain consistent biofilm formation among our three trials. Thus, we conclude that there is no correlation between biofilm production and erythromycin resistance in *E. coli* K30 wild-type, Δ*wza*, and Δ*wza-wzb-wzc* in liquid media.

## INTRODUCTION

*Escherichia coli* are known to be able to survive a multitude of different environments that range from soil to the human gut (1). Their ability to form capsules is a critical component of their ability to evade host defenses and survive in harsh environments (2). In *E. coli* K30, the Wzy cassette is responsible for export and assembly of extracellular polysaccharide capsules (2). The Wzy-dependent capsular polysaccharide export system consists of an outer membrane channel (Wza), inner membrane channel and tyrosine autokinase (Wzc), and regulatory phosphatase (Wzb) (2). Previous studies have shown that *wza*, *wzb*, and *wzc* are critical genes in the formation of group 1 capsules in *E. coli* K30 (2). Group 1 capsules consist of acidic polysaccharides, such as uronic acid, and these molecules play a role in mediating biofilm formation; however the presence of a capsule can also impede surface attachment (3).

Macrolides, such as erythromycin, are a class of antibiotics that act by binding the 50S ribosomal subunit in bacterial cells, and halt peptide synthesis by dissociating peptidyl-tRNA from the ribosome (4). Previous studies have found differing results between the wild-type and *wzy* cassette knockouts using Kirby-Bauer disc diffusion assays to compare the minimum inhibitory concentration (MIC) of *E. coli* K30 (wild-type), CWG281 (Δ*wza*), and CWG655 (Δ*wza-wzb-wzc*) *E. coli* K30 strains on solid media (5–8). A study by Botros *et al.* compared wild-type and Δ*wza-wzb-wzc E. coli* K30 strains against a variety of antibiotics, including erythromycin (5). They found that the K30 wild-type had a zone of inhibition whereas Δ*wza-wzb-wzc* did not, therefore concluding that the knockout mutation conferred resistance on solid media (5). Studies by Rana *et al.* and Chiu *et al.* found that

**Published Online:** September 2020

**Citation:** Cynthia James, Christine Kim, Catherine Pan, Douglas Zhong. 2020. Biofilm production in *Escherichia coli* K30 with group 1 capsular gene *wza* and *wza-wzb-wzc* deletions is not correlated with Erythromycin resistance phenotypes in liquid media. UJEMI 25:1-9

**Editors:** Daniela Morales and Julia Huggins, University of British Columbia



Address correspondence to: https://jemi.microbiology.ubc.ca/

wild-type *E. coli* K30 was the most susceptible to erythromycin, while *Δwza* and *Δwza-wzb-wzc* had similar levels of resistance on solid media (6, 7). Su *et al.* observed that wild-type was the most susceptible to erythromycin, followed by Δ*wza* and lastly Δ*wza-wzb-wzc*, which was the most resistant, using Kirby-Bauer disc diffusion assays (8). Overall, these studies found that Δ*wza* and Δ*wza-wzb-wzc* were more resistant to erythromycin than wild-type *E. coli* K30 on solid media.

Interestingly, subsequent studies noticed a different phenomenon occurred when liquid minimum inhibitory concentration (MIC) assays with Δ*wza*, Δ*wzc*, and Δ*wza-wzb-wzc E. coli* K30 strains were performed (8, 9). Jazdarehee *et al.* and Su *et al.* found that the macrolide-resistant phenotype of the knockouts was not observed when cultured in liquid media (8, 9). Jazdarehee *et al.* observed that the wild-type strain displayed similar resistance to the Δ*wza* strain in their MIC assay trials (9). Additionally, they observed inconsistent results in all three of their trials that compared MIC values between the wild-type and the Δ*wza* and Δ*wzc* strains in erythromycin (9). Furthermore, Su *et al.* observed that the Δ*wza-wzb-wzc* strain was equally as susceptible to erythromycin as wild-type *E. coli* K30 when cultured in liquid Luria-Bertani (LB) broth (8). The researchers noted that Δ*wza* was susceptible to half the concentration of erythromycin than both the wild-type and Δ*wza-wzb-wzc*; this was the opposite phenotype observed in solid media (8).

We suggest that the inconsistent phenotypes observed between solid and liquid media are due to biofilm formation. Biofilms are formed by the surface colonization of bacteria and are composed of bacteria, proteins, polysaccharide matrix, and DNA (10). The formation of bacterial biofilms requires the bacteria to adhere to a surface which can be difficult in a liquid culture due to hydrodynamic forces and limited surface area to adhere to (11). Biofilms help *E. coli* become more resistant to certain antibiotics through mechanisms such as inducing the expression of antibiotic resistance genes and having antibiotic-modifying enzymes in the biofilm (12, 13). Because the capsule can impede surface attachment of *E. coli* K30, knocking out the genes responsible for group 1 capsules could allow *E. coli* K30 to adhere to surfaces more strongly and produce biofilm—therefore accounting for the macrolide-resistant phenotype observed in previous studies (3).

Little research has been done on biofilm-mediated resistance to macrolides. Dhanoa *et al.* quantified the amount of biofilm produced in *E. coli* K12 wild-type, Δ*wza*, and Δ*wza-wzb-wzc* strains in the presence of streptomycin and cephaloridine (14). They found that in response to a sub-inhibitory concentration of streptomycin or cephaloridine, the wild-type cells produced the most biofilm, followed by the Δ*wza*, then Δ*wza-wzb-wzc* strains (14). Therefore, we were interested in observing if biofilm formation is correlated with the differential erythromycin-resistance phenotypes between solid and liquid media observed in previous studies. Jazdarehee *et al.* suggested that the regulator of capsule synthesis (Rcs) system may exhibit different activity on solid and liquid media, and thus account for the varying levels of capsule production in these two environments (9). Therefore, we hypothesized that the different erythromycin resistance phenotypes in *E. coli* K30 wild-type, Δ*wza*, and Δ*wza-wzb-wzc* strains are due to differences in biofilm production in solid and liquid media.

In this study, our aim was to investigate if biofilm production was correlated with differential erythromycin resistance phenotypes of *E. coli* K30 wild-type, Δ*wza*, and Δ*wza-wzb-wzc* strains in solid and liquid media. We performed Kirby-Bauer disc diffusion assays to determine erythromycin resistance on solid media, minimum inhibitory concentration (MIC) assay in liquid media to determine the liquid MIC, and crystal violet biofilm assays to quantify biofilm formation in all three strains. We observed that biofilm production does not correlate with differential erythromycin resistance phenotypes in solid and liquid media.

## METHODS AND MATERIALS

**Bacterial strain confirmation, media preparation and growth conditions.** *E. coli* K30 E69 (serotype: O9a:K30:H12), CWG281 (*wza*22min::*aadA wza*K30::*aacC1*), and CWG655 (*wza*22 min::*aadA* Δ(*wza-wzb-wzc*)K30::*aphA3*) were obtained from the MICB 447 laboratory stock from the Department of Microbiology and Immunology at the University of British Columbia. These strains were originally obtained from the laboratory of Dr. Chris Whitfield from the Department of Molecular and Cellular Biology at the University of

Guelph. In this paper, *E. coli* K30 E69 is referred to as the wild-type, CWG281 is referred to as *Δwza* and CWG655 as *Δwza-wzb-wzc*. Strains were verified by growing at 37℃ on antibiotic-supplemented Luria-Bertani (LB) agar media corresponding to the antibiotic resistance cassettes used to inactivate the genes (7). *Δwza* was grown on media supplemented with gentamicin (10 μg/mL) and spectinomycin (100 μg/mL) while *Δwza-wzb-wzc* was grown on media supplemented with kanamycin (100 μg/mL) and spectinomycin (100 μg/mL). In all experiments, liquid LB media was composed of 0.5% w/v NaCl, 1.0% w/v tryptone, and 0.5% w/v yeast. Solid LB agar had the same composition in addition to 1.5% agar. Liquid cultures were grown at 37℃ on a shaking incubator, and the plates were incubated at 37℃.

**Disc diffusion assay.** Using the Kirby-Bauer disc diffusion method modified by Chiu *et al.*, disc diffusion assays were completed for all three strains using 15 μg/mL erythromycin discs (7). The colony of each strain was inoculated in 5 mL of LB media for 18 hours at 37°C and shaking at 250 rpm. The following day, each overnight culture was diluted to 1 $OD_{660}$ in LB media, and 100 μL of the diluted cultures were streaked onto plates that contained exactly 20 mL of LB agar. Plates were filled using a serological pipette to ensure even thickness across all replicates. After the plates had dried, three 15 μg/mL erythromycin discs, obtained from the Department of Microbiology and Immunology at the University of British Columbia were placed evenly spaced apart on each plate using sterile forceps. Plates were incubated for 18 hours at 37°C. The next day, the radii of the zones of inhibition from the edge of the disc were measured.

**Minimum inhibitory concentration assay in liquid media.** A minimum inhibitory concentration (MIC) assay in liquid LB media was performed on all three strains based on a protocol by Su *et al.* (8). One colony of each strain was inoculated in 5 mL of LB media for 18 hours at 37°C and shaking at 250 rpm. The following day, each overnight culture was diluted to 0.01 $OD_{660}$. A 5mg/mL stock erythromycin solution was made by adding 0.05 g of erythromycin (Sigma) into 10 mL of 100% ethanol. The stock solution was then diluted with LB media to 500 ug/mL. In a tissue-culture treated 96-well plate (VWR 10861-666), 100 μL of LB media was added to columns 3 to 11 and 200 μL of 500 ug/mL erythromycin was added to column 2. 100 μL of the erythromycin in column 2 was then transferred to column 3, which contained 100 μL of LB media, and mixed together by pipetting up and down using new tips. 100 μL of the LB-erythromycin solution in column 3 was then transferred to column 4. This two-fold serial dilution was repeated up to column 10, where the 100 μL of solution from column 9 was discarded. The first and last wells of each row as well as the entire last row were filled with 200 μL of distilled water to minimize edge effects, a phenomenon where the wells on the edges of the plate evaporate quicker and result in inconsistent volumes among the wells (15). Row 1 contained no bacterial cells and acted as the negative control. To keep volumes consistent with the sample wells, 100 μL of LB media was added to the appropriate wells in row 1. 100 μL of wild-type *E. coli* K30 at 0.01 $OD_{660}$ was added to the appropriate wells in rows 2 and 3. 100 μL of diluted *Δwza* was added to the next two rows, followed by 100 μL of diluted *Δwza-wzb-wzc*. After incubation at 37℃ for 24 hours, the $OD_{660}$ was measured to determine the lowest concentration of erythromycin needed to inhibit bacterial growth in liquid culture, otherwise known as the liquid minimum inhibitory concentration.

**Biofilm assay.** Using the same 96-well plate that the liquid minimum inhibitory concentration (MIC) assay was completed in, a biofilm assay was carried out for all three strains. The biofilm assay was based off of the protocol by Merritt *et al.* (16). After $OD_{660}$ was measured for the MIC assay, the plate was incubated at 37°C for another 24 hours. The cells were then discarded, and the plate was washed two times with distilled water to remove unattached cells. Afterwards, 125 μL of 0.1% crystal violet was added to each well for 10-15 minutes. The plate was then washed three to four times in distilled water and vigorously blotted on a stack of paper towels to remove excess cells and dye. After drying the plate overnight, 125 μL of 30% glacial acetic acid was added to each well for 10-15

minutes to solubilize the crystal violet. 100 µL of the solubilized crystal violet was then transferred to a new 96-well plate, and the $OD_{550}$ was measured.

## RESULTS

**E. coli K30 strains Δ*wza* and Δ*wza-wzb-wzc* are more resistant to erythromycin than wild-type on solid media.** To confirm previous results that Δ*wza* and Δ*wza-wzb-wzc* were more resistant to erythromycin than wild-type *E. coli* K30 on solid media, we performed Kirby-Bauer disc diffusion assays on the three strains (5-8). Overnight cultures of wild-type, Δ*wza*, and Δ*wza-wzb-wzc* strains were diluted to 1 $OD_{660}$, then plated on solid LB media. Three 15µg/mL erythromycin discs were placed equidistant from each other on each plate, and the plates were incubated at 37°C for 18 hours. Following incubation, the zone of inhibition was measured from the edge of the erythromycin disc to where confluent growth began. This was repeated for five biological replicates for each strain. Figure 1 shows that wild-type exhibited the largest radius of inhibition (average 2.86 mm), Δ*wza* produced a smaller radius of inhibition (average 1.27 mm), and Δ*wza-wzb-wzc* displayed no radius of inhibition (average 0 mm). Due to the smaller radii of Δ*wza* and Δ*wza-wzb-wzc* compared to wild-type, we can confirm that Δ*wza* and Δ*wza-wzb-wzc* were indeed more resistant to erythromycin than wild-type. We also found that Δ*wza-wzb-wzc* was more resistant to erythromycin than Δ*wza* on solid media.

***E. coli* K30 erythromycin minimum inhibitory concentration is between 125 and 250 µg/mL in liquid media.** Previous literature did not find a consistent erythromycin minimum inhibitory concentration value for *E. coli* K30 wild-type, Δ*wza*, and Δ*wza-wzb-wzc* strains in liquid media (9). To investigate this, we performed a 96-well plate MIC serial dilution assay. Overnight cultures of wild-type, Δ*wza*, and Δ*wza-wzb-wzc* strains were diluted to 0.01 $OD_{660}$, then plated in duplicate at erythromycin concentrations of 0, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250, and 500 µg/mL in liquid LB media. The plate was incubated at 37°C for 24 hours, and optical density was measured at $OD_{660}$ to quantify cell growth. Water was added to the outer wells to prevent evaporation, and wells containing only LB media acted as our negative control to ensure our samples had no contamination.

Figure 2 shows that cell density decreases as erythromycin concentration increases and cell growth was strongly inhibited above 125 µg/mL of erythromycin for all strains. The negative control wells exhibited a consistent $OD_{660}$ reading (average 0.038) which meant that no contamination was present. Interestingly, Δ*wza* $OD_{660}$ readings were consistently lower than wild-type and Δ*wza-wzb-wzc* strains; however, throughout our experiments, we found that Δ*wza* grew slower than the other two strains. Therefore, we attributed the lower $OD_{660}$ readings at all concentrations to a lower rate of growth for this mutant. At 250 and 500 µg/mL of erythromycin, we found that all strains exhibited minimal growth across three

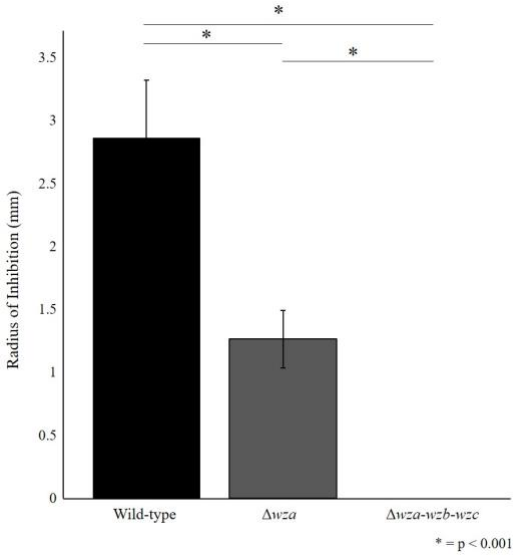


**FIG. 1 Δ*wza* and Δ*wza-wzb-wzc* are more resistant to erythromycin than wild-type on solid media.** Radius of inhibition was measured following a Kirby-Bauer disc diffusion assay using 15µg/mL erythromycin discs. Wild-type, Δ*wza*, and Δ*wza-wzb-wzc* strains were spread plated on solid LB agar. Three 15µg/mL erythromycin discs were then placed evenly spaced apart on each plate. All plates were incubated at 37°C for 18 hours, and the radius of inhibition was measured from the edge of the disc to where confluent growth began. Five biological replicates were tested for each strain. Error bars represent the standard deviation of 15 replicates. Student's paired t-test was performed between all strains. $\alpha = 0.05$.

biological replicates. Therefore, we concluded that the erythromycin minimum inhibitory concentration of *E. coli* K30 wild-type, Δ*wza*, and Δ*wza-wzb-wzc* strains was between 125 and 250 µg/mL in liquid media.

***E. coli* K30 biofilm production did not correlate with erythromycin concentration in wild-type, Δ*wza*, and Δ*wza-wzb-wzc* strains.** Finally, in order to test if biofilm formation was correlated with erythromycin concentration in liquid media, we performed a 96-well plate crystal violet staining assay. After measuring the cell density, shown in Figure 2, we placed the same 96-well plates back into the 37°C incubator for an additional 24 hours. After a total incubation time of 48 hours, we removed the media and planktonic bacteria from each well by submerging the 96-well plate in distilled water three times, then added 125µL of 0.1% crystal violet for 15 minutes. Crystal violet was removed by submerging the plate in distilled water for an additional three times, and left to dry at room temperature overnight. The crystal violet-stained biofilm was then solubilized with 30% glacial acetic acid for 15 minutes and transferred to a clean 96-well plate. The plate was read at $OD_{550}$ to quantify the biofilm produced. Water was added to the outer wells to prevent evaporation edge effects, and a negative control containing only LB media was used to ensure our samples had no contamination.

Figure 3A shows that for the wild-type, biofilm production decreased as the concentration of erythromycin increased, and no biofilm was produced at erythromycin concentrations above 15.6 µg/mL. For Δ*wza* and Δ*wza-wzb-wzc* strains, no biofilm was produced regardless of erythromycin concentration. Interestingly, Figure 3 (B) and (C) exhibited no distinct trends between biofilm formation and erythromycin concentration; no biofilm was detected at all concentrations of erythromycin for all strains. Our data indicates that in one of the three trials, the wild-type K30 strain was able to form low amounts of biofilm whereas in all three trials, the mutant strains did not form biofilm (Figure 3A, B, C). Therefore, this may indicate that wild-type is able to form biofilm in liquid media and the mutant strains are unable to form biofilm in similar conditions. Furthermore, resistance phenotypes observed in Figure 2 did not correlate with data in Figure 3. Thus, we conclude that biofilm formation is not correlated with erythromycin resistance for *E. coli* K30 wild-type, Δ*wza*, and Δ*wza-wzb-wzc* strains in liquid media, but we are unable to reach a conclusion on the relationship between erythromycin concentration and biofilm production in the same strains.

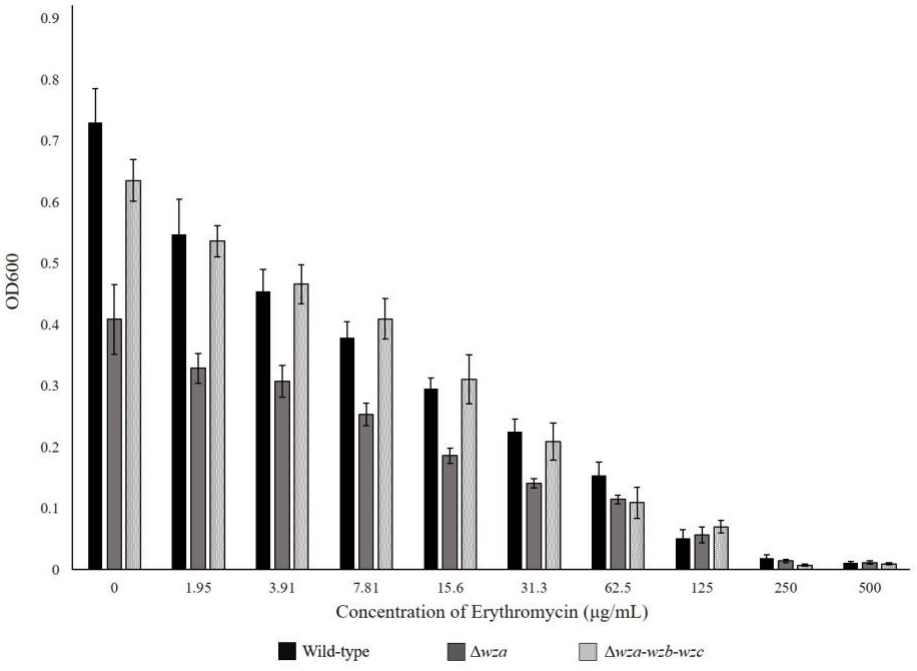


**FIG. 2 *E. coli* K30 erythromycin minimum inhibitory concentration is between 125 and 250 µg/mL in liquid media.** Cell density at erythromycin concentrations between 0 and 500 µg/mL was quantified using a 96-well plate minimum inhibitory concentration serial dilution assay. Wild-type, Δ*wza*, and Δ*wza-wzb-wzc* strains were plated in duplicate and grown at erythromycin concentrations of 0, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250, and 500 µg/mL for 24 hours at 37°C (n = 3). $OD_{660}$ was measured to quantify cell growth. $OD_{660}$ reading of pure LB control was subtracted from all samples while plotting. Error bars represent standard deviation of 10 technical replicates per strain.

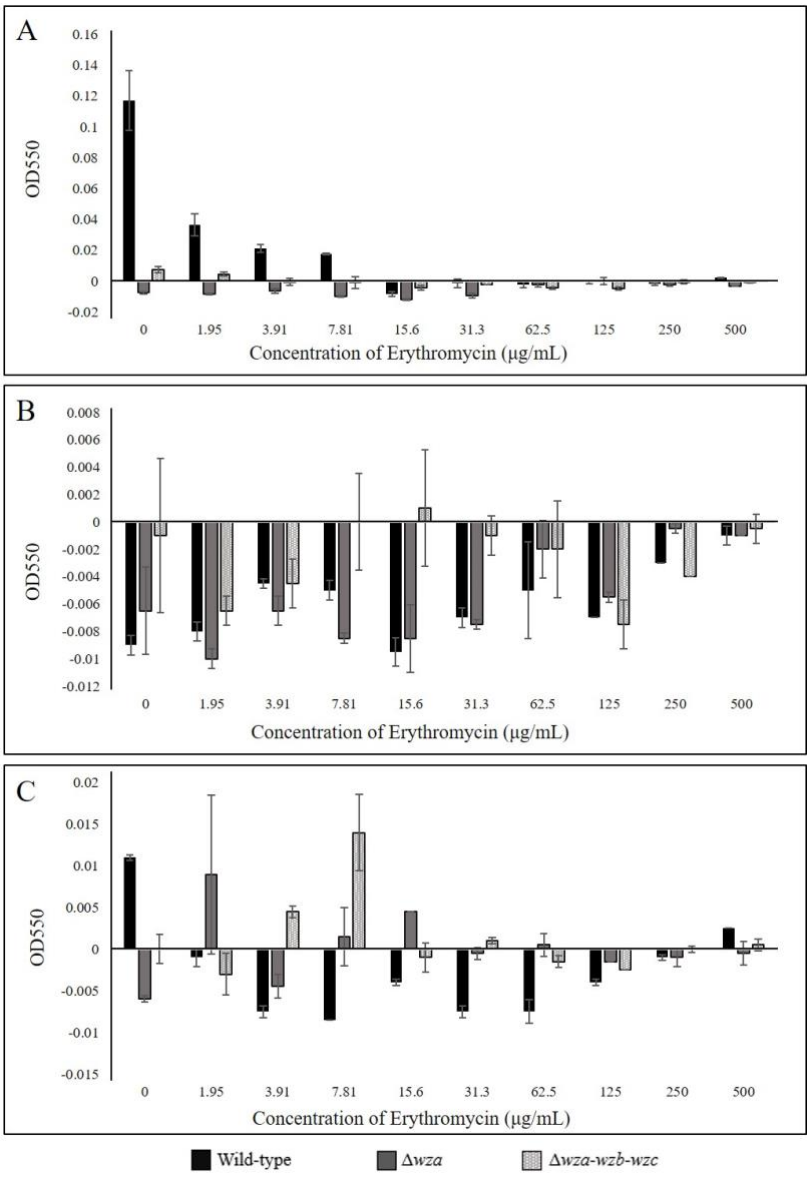


**FIG. 3 *E. coli* K30 biofilm production is not correlated with erythromycin resistance in wild-type, Δ*wza*, and Δ*wza-wzb-wzc* strains at 48 hours.** Biofilm production was quantified at $OD_{550}$ using a 96-well plate crystal violet staining assay after 48 hours of incubation at 37°C. Crystal violet was solubilized using 30% glacial acetic acid for 15 minutes, then transferred to a new 96-well plate for optical density reading at $OD_{550}$. $OD_{550}$ of pure LB control was subtracted from all samples while plotting. Panels (A), (B), and (C) represent one biological replicate each (n = 3). Error bars represent standard deviation for 2 technical replicates for each strain at each concentration.

## DISCUSSION

Our study investigated the relationship between the group 1 capsule transporter Wzy gene cassette (*wza*, *wzb*, *wzc*) and its effects on biofilm production in the presence of the macrolide erythromycin in *E. coli* K30. A previous study showed that Δ*wza* and Δ*wza-wzb-wzc* strains exhibited greater erythromycin resistance compared to the wild-type strain on solid media, but were less resistant to erythromycin in liquid media (8). Therefore, we hypothesized that the different erythromycin resistance phenotypes in *E. coli* K30 wild-type, Δ*wza*, and Δ*wza-wzb-wzc* strains were due to differences in biofilm production in solid and liquid media. To test this, we performed Kirby-Bauer disc diffusion assays to determine erythromycin resistance on solid media, a liquid MIC assay to investigate erythromycin resistance in liquid media, and a 96-well crystal violet staining assay to quantify biofilm produced. We found that Δ*wza* and Δ*wza-wzb-wzc* strains exhibited greater erythromycin resistance compared to the wild-type in solid media (Figure 1); however, all strains displayed similar resistance to erythromycin in liquid media (Figure 2). Furthermore, we found that there is no correlation between erythromycin resistance and biofilm production (Figure 3).

***Δwza* and *Δwza-wzb-wzc* are more resistant to erythromycin than wild-type on solid media.** The Kirby-Bauer disc diffusion assay results showed that the *E. coli* K30 wild-type

strain had the largest zone of inhibition, followed by Δ*wza*, and lastly Δ*wza-wzb-wzc* which had no zone of clearance (Figure 1). This suggested that the wild-type strain was the least resistant and the Δ*wza-wzb-wzc* strain was the most resistant to erythromycin on solid media. Botros *et al.* also observed that Δ*wza-wzb-wzc* was the most resistant (5). Conversely, Su *et al.* found that Δ*wza* was more resistant than Δ*wza-wzb-wzc* to erythromycin on solid media whereas Rana *et al.* found that both knock-out strains were equally resistant to erythromycin (6, 8). Furthermore, the sizes of the zones of inhibition for all strains were smaller in our observed results than those in previous literature. This could be due to the age of the erythromycin discs and improper storage conditions. Overall, we were unable to definitively determine which mutant strain was more resistant to erythromycin on solid media as there is conflicting literature; however, we confirmed that Δ*wza* and Δ*wza-wzb-wzc* strains were more resistant to erythromycin than the wild-type strain on solid media.

**Erythromycin resistance of wild-type, *Δwza*, and *Δwza-wzb-wzc* strains grown in liquid media differs from the resistance phenotypes on solid media.** Wild-type, Δ*wza*, and Δ*wza-wzb-wzc* had similar levels of erythromycin resistance in liquid media, and all growth was inhibited between 125 and 250 μg/mL of erythromycin (Figure 2). Therefore, we concluded that the MIC value for *E. coli* K30 wild-type, Δ*wza*, and Δ*wza-wzb-wzc* was between 125 and 250 μg/mL. Interestingly, this result contradicts with the Kirby-Bauer disc diffusion assay results where Δ*wza*, and Δ*wza-wzb-wzc* were found to be more resistant to erythromycin compared to the wild-type. Furthermore, liquid media results of previous studies differed from what we observed: Jazdarehee *et al.* were unable to find a consistent MIC value for any of the three strains tested, and Su *et. al.* found that the Δ*wza* mutant had a MIC value of 125 μg/mL and both the wild-type and Δ*wza-wzb-wzc* strains had a MIC value of 250 μg/mL (8, 9). The differences between past literature MIC values and our MIC value may be attributed to protocol differences. Previous research plated very dilute culture (0.0001 $OD_{660}$), whereas we plated our overnight culture at 0.01 $OD_{660}$ (8, 9). Plating with a denser culture ensured that an equal number of cells were seeded per well, and this may have contributed to the consistent cell density readings for each concentration of erythromycin observed between trials.

**No correlation between biofilm production and erythromycin resistance for wild-type, *Δwza*, and *Δwza-wzb-wzc* strains in liquid media.** To quantify biofilm production in the presence of erythromycin, we performed a 96-well plate crystal violet staining assay at 37°C. However the optimal incubation time was unknown. Therefore, we performed this assay at 48 and 72 hours (Figure 3, Supplemental Figure 1). Because we found no significant difference in biofilm production between time points, we analyzed biofilm production at 48 hours due to time constraints. We were unable to obtain consistent biofilm production across three trials (Figure 3). Figure 3 (A) exhibited a distinct negative relationship between biofilm produced and erythromycin concentration for the wild-type strain, but no biofilm was detected for Δ*wza* or Δ*wza-wzb-wzc* mutants in that trial. Conversely, Figure 3 (B) and (C) did not show biofilm production for any strains at all erythromycin concentrations. Therefore, our data displayed two conflicting results: biofilm is correlated with erythromycin concentration in wild-type *E. coli* K30 (Figure 3A), or biofilm is not correlated with erythromycin concentration for all strains (Figure 3B, C).

Biofilm quantification was performed at 37°C, however the optimal incubation time was unknown. Therefore, we performed this assay at 48 and 72 hours (Figure 3, Supplemental Figure 1). Because we found no significant difference in biofilm production between timepoints, we analyzed biofilm production at 48 hours due to time constraints. While we observed a negative correlation between biofilm production and erythromycin concentration in the wild-type strain for one trial (Figure 3A), we were unable to reproduce these results in our subsequent trials (Figure 3B, C). However, in all trials, regardless of whether biofilm was formed or not, there was no change in the level of erythromycin resistance in liquid media for all three strains. Therefore, there is no correlation between biofilm formation and erythromycin resistance in liquid media for *E. coli* K30 wild-type, Δ*wza*, and Δ*wza-wzb-wzc*. However, the inconsistent biofilm formation can be due to non-

optimal temperature and pH, which are important factors for biofilm formation (17). Furthermore, we performed the biofilm assay in 96-well tissue-culture treated plates. These plates may have resulted in inconsistent bacterial attachment; surface attachment is critical for biofilm formation. However, due to time constraints, we were unable to perform an attachment assay to determine how well the bacteria attached to the tissue-culture treated plates in comparison to non-tissue-culture treated plates. In addition, we used crystal violet staining which, although is a simple and sensitive method for biofilm quantification, has several limitations. Notably, it stains only the extracellular polysaccharide, but biofilm is composed of proteins, DNA, and polysaccharides (10, 15). Thus, with crystal violet staining, we were unable to measure the total amount of biofilm formed.

**Conclusions** Here we show that *E. coli* K30 Δ*wza* and Δ*wza-wzb-wzc* strains exhibit an erythromycin resistant phenotype on solid media. Furthermore, we determined that there is no difference in erythromycin susceptibility between wild-type, Δ*wza*, and Δ*wza-wzb-wzc* strains in liquid culture, and the MIC for these strains is between 125 and 250 μg/mL. Finally, we were unable to observe a direct correlation between biofilm production and erythromycin resistance in liquid media for *E. coli* K30 wild-type, Δ*wza* and Δ*wza-wzb-wzc* strains.

**Future Directions** In addition to wild-type *E. coli* K30, Δ*wza*, and Δ*wza-wzb-wzc*, previous papers studied the erythromycin resistance of Δ*wzc* and found inconsistent results in solid and liquid media. Chiu *et al*. found that Δ*wzc* was more susceptible to erythromycin compared to Δ*wza* and Δ*wza-wzb-wzc* but was more resistant than the wild-type (7). In contrast, Jazdarehee *et al*. found that Δ*wzc* was more resistant than Δ*wza* on solid media, but was not able to find a distinct MIC value for Δ*wzc* in liquid media (9). Due to these inconsistent results in liquid and solid media, we initially planned to test Δ*wzc* in addition to the three strains, however we were unable to obtain the Δ*wzc* strain. Thus, a future study comparing the relative erythromycin resistance of the wild-type, Δ*wza*, Δ*wza-wzb-wzc*, and Δ*wzc* in solid and liquid media will be valuable. In addition, investigating whether there is a relationship between biofilm formation in liquid media and erythromycin resistance for Δ*wzc* will also be worthwhile. Here, we did not observe a correlation between biofilm production and erythromycin resistance for the wild-type, Δ*wza* and Δ*wza-wzb-wzc* as we were unable to form consistent biofilm between trials. Repeating the biofilm assay using different temperatures and incubation times to determine whether consistent biofilm can be formed will be useful. Botros *et al*. found that Δ*wza-wzb-wzc* had lower capsular polysaccharide production compared to the wild-type at 21°C but not at 37°C (5). Thus, it would be interesting to find out whether varying temperatures will have an effect on the amount and level of consistency of biofilm formation. If biofilm is inconsistent, discovering the factor that leads the differential phenotypes in liquid and solid media will be valuable.

In this study, we performed the crystal violet biofilm assay in 96-well tissue-culture treated plates. We suggest that inconsistent biofilm production may be related to inconsistent bacterial attachment to the wells of the tissue-cultured plate compared to non-tissue-cultured plates; surface attachment is critical for biofilm production. Due to time constraints, we were unable to perform an attachment assay to determine how well the bacteria attached to the tissue-culture treated plates, but it would be a beneficial test to carry out in the future. We also used crystal violet staining to measure the amount of biofilm produced. This method is widely used due to its simplicity and sensitivity, but it has several limitations that should be taken into consideration when designing future experiments (18). Biofilm is composed of proteins, DNA and polysaccharides (10). However, crystal violet only stains the extracellular polysaccharide (EPS) present in biofilms, and thus does not measure total biofilm formation (10, 15). Furthermore, with crystal violet staining, some of the steps prior to quantification such as washing may lead to loss of biofilm (18). Other more specific and reproducible methods to measure biofilm production include using light microscopy to directly visualize the biofilm structures (19). Using microscopy eliminates the need to resuspend the bacteria which prevents biofilm disruption and subsequent destruction.

## ACKNOWLEDGEMENTS

We would like to thank the Department of Microbiology and Immunology at the University of British Columbia for the funding and opportunity to investigate this project. We are grateful to Dr. David Oliver and Dr. Evelyn Sun for their continual guidance, support, and feedback. We would also like to thank the UBC Biological Sciences Building media room staff for their assistance with reagents and lab equipment. We would also like to thank the Whitfield Lab for providing the strains. Lastly, we are thankful to our peers for collaborating with us by providing valuable feedback and sharing reagents.

## CONTRIBUTIONS

All authors contributed equally in writing this paper.

## REFERENCES

1. **van Elsas JD, Semenov AV, Costa R, Trevors JT**. 2011. Survival of *Escherichia coli* in the environment: fundamental and public health aspects. ISME J 5:173–183.
2. **Whitfield C**. 2006. Biosynthesis and Assembly of Capsular Polysaccharides in *Escherichia coli.* Annu. Rev. Biochem 75:39–68.
3. Rahn A, Whitfield C. 2003. Transcriptional organization and regulation of the *Escherichia coli* K30 group 1 capsule biosynthesis (*cps*) gene cluster. Mol Microbiol 47:1045–1060.
4. **Menninger JR, Otto DP**. 1982. Erythromycin, carbomycin, and spiramycin inhibit protein synthesis by stimulating the dissociation of peptidyl-tRNA from ribosomes. Antimicrob Agents Chemother 21:811–818.
5. **Botros S, Mitchell D, Ommen CV**. 2015. Deletion of the *Escherichia coli* K30 Group I Capsule Biosynthesis Genes *wza*, *wzb* and *wzc* Confers Capsule- Independent Resistance to Macrolide Antibiotics. J Exp Microbiol Immunol 19:8.
6. **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. J Exp Microbiol Immunol 20:6.
7. **Chiu J, Han G, McCrystal K, Zuo M**. 2017. Macrolide Structures Can Confer Differential Susceptibility in *Escherichia coli* K30 Deletions of Group 1 Capsule Assembly Genes. J Exp Microbiol Immunol 3:7.
8. **Su A, Wang, A, Yeo L**. 2017. Deletion of the Group 1 Capsular Gene *wza* in *Escherichia coli* E69 Confers Resistance to the Antibiotic Erythromycin on Solid Media but not in Liquid Media. J Exp Microbiol Immunol 3:8.
9. **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. J Exp Microbiol Immunol 21:5.
10. **Beloin C, Roux A, Ghigo J-M**. 2008. *Escherichia coli* biofilms. Curr Top Microbiol Immunol 322:249–289.
11. **Sharma G, Sharma S, Sharma P, Chandola D, Dang S, Gupta S, Gabrani R**. 2016. *Escherichia coli* biofilm: development and therapeutic strategies. J. Appl. Microbiol. 121:309–319.
12. **Hall CW, Mah T-F.** 2017. Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. FEMS Microbiol Rev 41:276–301.
13. **Singh S, Singh SK, Chowdhury I, Singh R**. 2017. Understanding the Mechanism of Bacterial Biofilms Resistance to Antimicrobial Agents. Open Microbiol J 11:53–62.
14. **Dhanoa T, Li WWY, Thomas K, Wu B**. 2015. Sub-minimum Inhibitory Concentration of Streptomycin and Cephaloridine-Induced Capsular Polysaccharide Production in *Escherichia coli* K-12 Increases Biofilm Formation in a Wzy-transporter Dependent Manner. J Exp Microbiol Immunol 19.
15. **Shukla SK, Rao TS**. 2017. An Improved Crystal Violet Assay for Biofilm Quantification in 96-Well Microtitre Plate. bioRxiv 100214.
16. **Merritt JH, Kadouri DE, O'Toole GA**. 2005. Growing and Analyzing Static Biofilms. Curr Protoc Microbiol 0 1:Unit-1B.1.
17. **Mathlouthi A, Pennacchietti E, De Biase D**. 2018. Effect of Temperature, pH and Plasmids on In Vitro Biofilm Formation in *Escherichia coli.* Acta Naturae 10:129–132.
18. **Burton E, Yakandawala N, LoVetri K, Madhyastha MS**. 2007. A microplate spectrofluorometric assay for bacterial biofilms. J Ind Microbiol Biotechnol 34:1–4.
19. **Wilson C, Lukowicz R, Merchant S, Valquier-Flynn H, Caballero J, Sandoval J, Okuom M, Huber C, Brooks TD, Wilson E, Clement B, Wentworth CD, Holmes AE**. 2017. Quantitative and Qualitative Assessment Methods for Biofilm Growth: A Mini-review. Res Rev J Eng Technol 6.