Volume 6: 1-15

UJEMI+

The Undergraduate Journal of Experimental Microbiology & Immunology (+Peer Reviewed)

Deletions in the capsular polysaccharide *wzy* Cassette Genes differentially affect susceptibility to Nitrofurantoin in *Escherichia coli* K-12 compared to *Escherichia coli* K30

Rehanna Thobani, Gillian Savage, Malhar Shah

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

SUMMARY The *wzy* cassette is a set of genes responsible for capsular polysaccharide and exopolysaccharide polymerization and export. It has been shown that knockouts of the *wzy* cassette in *Escherichia coli* K30 strain increases susceptibility to the antibiotic nitrofurantoin, but a similar effect has not been observed in *E. coli* K-12. Based on this, we hypothesized that deletion of genes within the *wzy* cassette would change the susceptibility to nitrofurantoin differentially in *E. coli* K-12 compared to *E. coli* K30. To test this, susceptibilities to nitrofurantoin of mutants with knockouts in the *wzy* cassette compared to wildtypes were determined with disc diffusion assays at 28℃ and 37℃, as well as through a minimum inhibitory concentration (MIC) microtitration assay. Furthermore, the presence of capsule in all variants was determined with India ink staining to investigate the role of capsule in nitrofurantoin resistance. Our results show an increase in susceptibility to nitrofurantoin in only the *E. coli* K30 *wzb* and full *wzy* cassette knockouts at 37℃, but an increase in resistance in the *E. coli* K-12 *wza* knockout at 28℃. The MIC assay in liquid media showed the MIC for *E. coli* K30 *wza* was higher than the MIC of the *E. coli* K30 wildtype whereas the *wzb* and full cassette knockouts showed no change. Furthermore, capsule was only visualized in the *E. coli* K30 wildtype. Altogether our results suggest that the *E. coli* K30 and K-12 *wzy* cassette mutants respond differentially to nitrofurantoin and this effect occurs through a capsule-independent mechanism.

INTRODUCTION

Urinary tract infections (UTIs) are some of the most common bacterial infections worldwide, affecting over 150 million people annually (1, 2). Nitrofurantoin is a broad-spectrum antibiotic in the class of nitrofurans being prescribed at increasingly higher rates to treat UTIs for both Gram-positive and Gram-negative infections (3). Though the mechanism of action is not well described, the generally accepted theory is that upon entry into the cell, nitrofurantoin is converted by bacterial reductases into an active form that damages proteins, DNA and RNA (4). Despite very low rates of resistance in practice, the present model for resistance to nitrofurantoin includes mutations in the bacterial reductases *nsfA* and *nsfB*, resulting in an impaired ability to reduce, thus activate, nitrofurantoin (3-5).

The *wzy* cassette is an operon which consists of the highly conserved genes *wza, wzb* and *wzc* (6-8). Type 1 capsular polysaccharides (CPS) as well as exopolysaccharides (EPS) are synthesized in a Wzy-dependent pathway, named for its dependence on the polymerase Wzy (6, 9). These genes are involved in the expression of CPS on the cell surface in conjunction with other proteins. WbaP, a transferase, initiates CPS production (10). The product is then glycosylated by WbaZ, WcaO and WcaN (7). The glycosylated product is transported across the inner membrane by the flippase Wzx, then polymerized by Wzy (7). Wzb is a

Published Online: September 2020

Citation: Rehanna Thobani, Gillian Savage, Malhar Shah. 2020. Deletions in the capsular polysaccharide *wzy* Cassette Genes differentially affect susceptibility to Nitrofurantoin in *Escherichia coli* K-12 compared to *Escherichia coli* K30. UJEMI+ 6:1-15

Editor: Daniela Morales and Julia Huggins, University of British Columbia

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

phosphotyrosine phosphatase which interacts with Wzc, an inner membrane tyrosine autokinase, to control the degree of CPS polymerization (Fig. 1) (11-13). Wzc spans the inner membrane and contains a periplasmic domain that interacts with Wza, a translocon situated on the outer membrane (Fig. 1) (9, 14). Once CPS is formed, the interaction between Wzc and Wza initiates translocon activity and the polymer is exported across the outer membrane (6). Once exported, the polysaccharide associates with the outer membrane lectin Wzi that allows the formation of capsule (15).

In *Escherichia coli* K30*,* Type 1 CPS form a K-antigen capsule that is important during infections since capsules facilitate adherence, allow moisture retention, and provides protection from the host response and antimicrobial peptides (6). It has been shown that deletion of *wza, wzb* and *wzc* all result in an acapsular phenotype, therefore the genes within the *wzy* cassette are necessary for capsule synthesis in *E. coli* K30 (15).

E. coli K-12 also contains the *wzy* cassette (8). However, unlike *E. coli* K30, *E. coli* K-12 has some genetic changes in its polysaccharide synthesizing genes and lacks *wzi*, and therefore does not produce K capsule (10, 17, 18). Instead, *E. coli* K-12 produces colanic acid, a loosely-associated, negatively-charged EPS secreted into the extracellular space (16). Colanic acid is important for biofilm synthesis and survival within the environment and is produced only in very small amounts above 30℃, unlike K capsule synthesis (8, 17). These genes in *E. coli* K-12 can be expressed when the bacteria are subjected to stressors such as low temperatures, osmotic shock or overexpression of particular proteins (16). Despite these differences, the *wzy* cassette genes in both strains are conserved such that knockouts in *E. coli* K30 within the cassette can be complemented with the genes from K-12 allowing the bacteria to maintain capsule production (9).

In previous studies, the *E. coli* K30 strain CWG655 Δ[*wza-wzb-wzc*] exhibited an increased susceptibility to nitrofurantoin compared to the wildtype (WT) strain (6, 7). Gu *et al.* also demonstrated that a single gene knockout of *wzb* within the cassette (Δ*wzb)* increased susceptibility to nitrofurantoin while a Δ*wza* strain CWG281 exhibited no difference in resistance (7). In the same study, *E. coli* K-12 strain JW2046-1 (Δ*wzb*) did not exhibit increased susceptibility compared to the WT K-12, but rather showed slightly increased resistance (7). This suggests a differential role of the *wzy* cassette components in nitrofurantoin resistance in *E. coli* K30 compared to *E. coli* K-12. However, these differences have not been explored and the mechanism of nitrofurantoin resistance in these two bacterial strains still remains fairly unknown. We hypothesize that the *wzy* cassette plays a differential role in nitrofurantoin resistance in *E. coli* K-12 compared to *E. coli* K30. To assess this, we evaluated the susceptibility to nitrofurantoin in *E. coli* K30 and *E. coli* K-12 strains with selective knockouts for either *wza*, *wzb*, or the full *wzy* cassette at temperatures of 37℃ or 28℃. We then further imaged these strains for capsule to investigate if the presence of capsule is an important component of the resistance mechanism.

TABLE. 1 *E. coli* strains used in this study

Strain	Genotype	Antibiotic Resistance Marker
E69	K30 WT	N/A
CWG281	K30 E69 with $wza_{22\ min}$*::aadA, Δwza*	Gentamicin
CWG343	K30 E69 with $wza_{22\ min}$*::aadA, Δwzb*	Kanamycin
CWG655	K30 E69 with $wza_{22\ min}$*::aadA, Δ(wza-wzb-wzc)*	Kanamycin
BW25113	K-12 WT	N/A
JW2047-1	BW25113 K-12 with Δ*wza -760::kan*	Kanamycin
JW2046-1	BW25113 K-12 with Δ*wzb-759::kan*	Kanamycin

METHODS AND MATERIALS

Bacterial strains, media, and growth conditions. *E. coli* bacterial strains used within this study are listed in Table 1. The *E. coli* K30 strains E69, CWG281, and CWG343 were obtained from the laboratory of Dr. Chris Whitfield from the Department of Molecular and Cellular Biology, University of Guelph and stored at the University of British Columbia as frozen glycerol stocks. The *E. coli* K-12 strains BW25113, JW2047-1, and JW2046-1 were originally obtained from the Yale Coli Genetic Stock Center as part of the Keio collection, and were stored and obtained from the Microbiology and Immunology department at the University of British Columbia. All strains were grown up on Lysogeny broth (LB) agar supplemented with 100µg/mL of appropriate antibiotic resistance marker, specified for each strain in Table 1, at 37℃ for 18-22 hours. Plates were stored at 4℃ until use. Overnight cultures were grown in either LB, for PCR, or Mueller Hinton (MH) media, for disc diffusion and MIC assays, at 37℃ or 28℃ for 16 to 20 hours with shaking at 200rpm.

PCR and gel electrophoresis to confirm strains. Overnight cultures were subject to crude DNA extraction, in which 30µl of culture was centrifuged for 30 seconds, and the pellet was resuspended in 50µl of sterile water. Bacterial suspension was incubated in a 96℃ water bath for 5 minutes, before centrifugation at max speed for 1 minute. Supernatants were used in PCR reactions as template DNA. *E. coli* strains were verified using PCR primers listed in Table 2. Each PCR reaction was set up as follows: 5µl of 10x PCR buffer (no $MgCl_2$, Invitrogen, Cat #18067017), 1.5µl of 50mM $MgCl_2$, 1µl of 10mM dNTP mix, 0.2µl of Platinum™ Taq Polymerase (Invitrogen, Cat #10966034), 1µl template DNA, 1µl of 10µM forward primer, 1µl of 10µM reverse primer, and 39.3µl of sterile dH_2O. Primers used are listed in Table 2. pUC19-193F and pUC19-355R primers were used as a positive PCR control with pUC19 plasmid as template. Negative controls were set up with sterile dH_2O instead of template for each primer set. PCR reactions were run on the Bio-Rad T100™ Thermal Cycler as following: 94℃ for 120 seconds, followed by 35 cycles of 94℃ for 30 sec, 50℃ for 30 sec, 72℃ for 4 min 50 seconds, and completed with a final extension of 72℃ for 10 minutes. PCR products were kept overnight in the thermocycler at 4℃. The resulting PCR products with 6X DNA loading dye and MassRuler Express Forward DNA Ladder (ThermoFisher Scientific, Cat #SM1283) were run on a 0.8% agarose gel made with SYBR® Safe DNA Gel Stain in 0.5X TBE (Invitrogen, Cat #S33100) at 100V for 45 minutes. Gel was visualized under UV light on the ChemiDoc™ MP Imaging System from Bio-Rad.

Disc diffusion assay. Isolated colonies from each strain were inoculated and grown overnight in MH media at 37℃ or 28℃. The overnight cultures were diluted with sterile MH media to an optical density at 660nm wavelength (OD_{660}) of 1.0. 200µl of each culture was spread onto MH agar plates, and four Oxoid™ 300µg nitrofurantoin susceptibility discs (ThermoFisher Scientific, Cat #CT0036B) were placed on each plate using sterile forceps. Plates were

TABLE. 2 Primer sequences for *PCR*

Primer	Nucleotide Sequence (5' to 3')
K30 *wzabc* Forward (EB6)	ggtcagggatccaacagtctg
K30 *wzabc* Reverse (EB7)	tcgcggatcaattgttacga
pUC19-193F	gtgaaataccgcacagatgc
pUC19-355R	ggcgttacccaacttaatcg
K-12 *wzabc* Forward	tattctgtcaatagcctgcgg
K-12 *wzabc* Reverse	tccaggtcggcatatagattg

allowed to grow at 37℃ or 28℃ for 18 to 20 hours, before diameters of the inhibition zones were measured in mm.

MIC assay. Isolated colonies from each strain were inoculated and grown overnight in 3ml MH media, shaking at 37℃ at 200rpm. The overnight cultures were diluted with sterile MH media to an OD_{600} of 0.1. Two-fold serial dilutions in MH media of nitrofurantoin (diluted in MH media from a 30mg/ml stock), ranging from 40-2.5µg/ml or 60-3.75µg/ml, were performed in microfuge tubes. 50µl per well of nitrofurantoin dilutions was added to separate rows of a 96-well plate. 50µl of each strain was added in duplicates into each nitrofurantoin dilution. To confirm the growth of each strain, even in the presence of dimethylformamide (DMF), duplicate wells were set up containing 50µl of cells combined with 50µl of MH media containing the same amount of DMF found in the 40µg/ml nitrofurantoin dilution. A standard for normalization of the data was set up combining 50µl of each nitrofurantoin dilution with 50µl of MH media. Four columns of 100µl of MH media were used as blanks and sterility controls. The plate was placed in the Biotek Epoch^{TM} 2 microplate spectrophotometer set up with the following conditions: total time: 16 hours, measurement interval: 10 minutes, primary wavelength: 600nm, temperature set point: 37^{o}C, continuous orbital shake at low speed.

India Ink Staining. Strains were streaked out on MH agar plates and grown overnight for 16-18 hours at either 37^{o}C or 28^{o}C. A drop of sterile water was placed on a glass slide, then an isolated colony was mixed in the water. 2µl of India ink was then added to the mixture. A #1.5 glass cover was then placed on top of the liquid, creating a wet mount. The samples were imaged with phase contrast under an Olympus BX53 Light/Fluorescence upright microscope with a 40x/NA 0.75/Air U Plan Fluorite Objective (400X magnification).

Statistical Analysis. All statistical analysis was performed in the open source computer environment R. Disk diffusion data was found to be non-normal and the non-parametric Kruskal-Wallis test was performed to look at differences across strains. Post hoc multiple comparisons were performed by applying the Wilcoxon ranked sum test with Bonferroni corrected p-values.

TABLE. 3 Summary of differences in nitrofurantoin susceptibility compared to wildtype for each knockout

Knockout Strain	Disk Diffusion Assay		MIC Assay
	37^{o}C	**28**^{o}C	**37**^{o}C
	***E. coli* K30**		
wzy full cassette	Increased susceptibility	Increased	Unchanged
wza	Unchanged	Increased	Decreased
wzb	Increased	Increased	Unchanged
wzc		Not tested	
	***E. coli* K-12**		
wzy full cassette		Not tested	
wza	Unchanged	Decreased	Unchanged
wzb	Unchanged	Unchanged	Unchanged
wzc		Not tested	

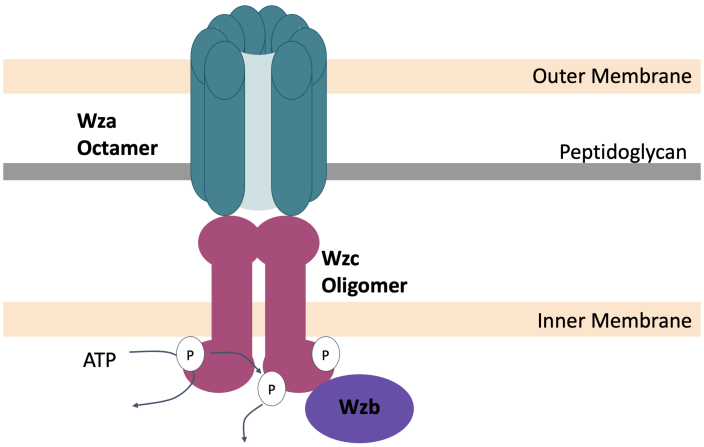

FIG. 1 Schematic drawing of *wzy* cassette products. Wza is an outer membrane translocon through which capsular polysaccharides are exported. Wzb is a phosphotyrosine phosphatase located on the cytoplasmic side of the membrane that interacts with Wzc. Wzc is a tyrosine autokinase that spans the inner membrane and interacts with Wza via its periplasmic domain.

RESULTS

Genotypes of wildtype and *wzy* gene knockouts in *E. coli* K-12 and K30 strains were confirmed by PCR and gel electrophoresis. To confirm the genotype of each *E. coli* K-12 and K30 knockout strain, genomic DNA was extracted and the entire *wzy* cassette was amplified by PCR and analyzed by agarose gel electrophoresis. EB6 and EB7 primers were used to amplify the cassette in the *E. coli* K30 samples while we designed primers for the K-12 samples. (Table 2.) pUC-19 plasmid amplified with pUC19-193F and pUC19-355R primers was used as a positive control for the PCR. As a negative control each of the primer sets were added to dH_2O instead of template. Products from *E. coli* K30 E69 (WT) and K-12 BW25113 (WT) had an expected band size of approximately 4200bp (Fig. S1A). The amplicon from the full *wzy* cassette knockout K30 CWG655 showed a band between 500 and 700 bp. This was expected as the cassette was deleted and replaced with a kanamycin resistance cassette (18). Products from the single gene knockouts were expected to yield a product greater than 4200bp as the deletions were made with an insertion of antibiotic resistance gene markers (18). As seen in Figure S1A, the *E. coli* K-12 JW2046-1 (Δ*wzb), E. coli* K30 CWG281 (Δ*wza*), *E. coli* K30 CWG343 (Δ*wzb*), *E. coli* K-12 JW2047-1 (Δ*wza)* bands look to be slightly larger than WT (Fig. S1B). The negative control lanes were clear of any product and a faint band was observed in the positive control lane. Faint bands at below 500bp, likely non specific products, were observed in each of the K30 lanes. Altogether, the results from the gel electrophoresis were as expected and gave us confidence in the identity of each of the mutant strains.

***wzy* cassette and *wzb* knockouts in *E. coli* K30 are more susceptible to nitrofurantoin while knockouts in *wza* and *wzb* do not affect susceptibility in *E. coli* K-12 at 37°C on solid media.** In order to determine the effect of *wzy* cassette knockouts on susceptibility to nitrofurantoin, disc diffusion assays were performed. Strains were cultured overnight in MH broth at 37°C and diluted to 1.0 $O.D_{600}$ before being plated on MH agar with four 300µg nitrofurantoin discs. Plates were incubated at 37°C overnight and zones of inhibition (ZOI) were measured the following day. CWG6555 Δ*(wza-wzb-wzc)* and CWG343 (Δ*wzb*) had larger zones of inhibition indicating that they were significantly more susceptible to nitrofurantoin than the E69 WT strain (mean zone of inhibition 29.6, 26.3 and 22.1mm respectively) (Fig. 2). Differences in zones of inhibition between CWG281 (Δ*wza*) (mean ZOI=21.7mm) and E69 were not statistically significant. CWG6555 was also found to be significantly more susceptible than CWG343, since the average zone of inhibition diameter was a few millimeters wider. There was no statistically significant difference in susceptibility across the K-12 strains with the zones of inhibition being of comparable size (Fig. 3). *E. coli* K-12 BW25113 (WT), JW2047-1 (Δ*wza)*, and JW2046-1 (Δ*wzb*) had zones of inhibition with a mean diameter of 20.02, 21.04 and 21.1mm respectively. The K30 wildtype strain had significantly larger zones of inhibition than the K-12 wildtype at 37°C. (bonferroni corrected P=0.023). Taking these results together we can conclude that at 37°C in *E. coli* K30, knocking

out *wzb* but not *wza* is sufficient to increase nitrofurantoin susceptibility, but there is no effect on susceptibility when the same genes are knocked out in *E. coli* K-12.

Knocking out *wza* in *E. coli* K30 increases the minimum inhibitory concentration (MIC) of nitrofurantoin in liquid media while in K-12 knockouts of *wza* and *wzb* do not alter nitrofurantoin MIC. In order to elucidate more specifically how knockouts in *wzy* genes affects susceptibility to nitrofurantoin, a growth curve assay was conducted to determine minimum inhibitory concentrations (MICs) of nitrofurantoin for each of the wildtypes and knockouts. Strains were grown overnight in MH broth at 37°C in a shaker incubator before being diluted to 0.1 $O.D_{660}$ and plated in duplicates in a 96-well plate with equal volume nitrofurantoin diluted in two-fold serial dilutions in MH broth ranging from 40-2.5µg/ml (trial 1) and 60-3.75µg/ml (trial 2). As the nitrofurantoin was reconstituted in DMF, for the 0µg/ml nitrofurantoin condition each of the strains were plated with DMF diluted in MH media to the same concentration of DMF present in the least dilute antibiotic solution. Wells with each of the nitrofurantoin concentrations in MH broth only were used as normalization standards. Four wells with MH media alone were used as sterility controls and as blanks. The plate was incubated overnight in the plate reader at 37°C on a continuous shake with readings taken every 10 minutes to generate growth curves for each of the conditions. Figure 4 shows growth curves for each of the strains obtained from the second trial, which are also representative of the first trial (Figure S2). No growth was observed in the sterility controls and bacteria in the 0µg/ml nitrofurantoin treatment grew similarly to WT strains that were plated in media only (Data not shown). *E. coli* K30 strains E69 (WT), CWG6555 Δ*(wza-wzb-wzc)* and CWG343 (Δ*wzb*) all had an estimated MIC of 30µg/ml with the E69 appearing to grow faster and to a higher concentration at stationary phase. CWG281 (Δ*wza*) was able to grow at nitrofurantoin concentrations up to 40µg/ml and had an estimated MIC of 60µg/ml. *E. coli* K-12 strains BW25113 (WT), JW2047-1 (Δ*wza*), and JW2046-1 (Δ*wzb*) all grew at similar rates and to similar concentration at stationary phase (Fig. 4). The estimated MIC of nitrofurantoin for all

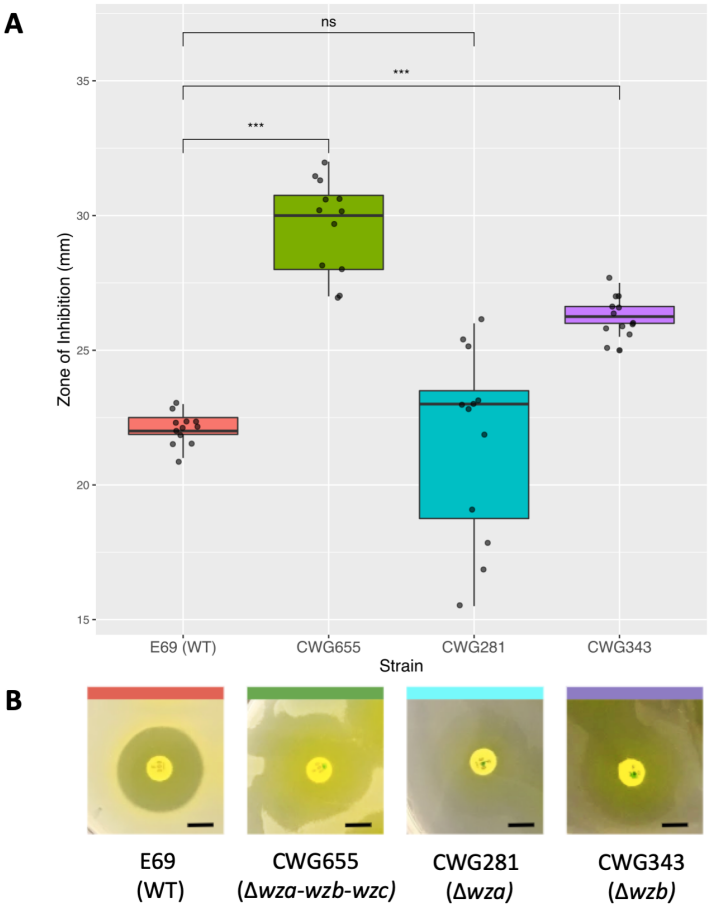

FIG. 2 Deletion of *wzb* or full *wzy* cassette results in larger zones of inhibition by nitrofurantoin in *E. coli* K30 at 37°C on solid media. (A) Box plot representation of results. Boxes span the interquartile range and dark line indicates the median value. Whiskers represent largest and smallest non-extreme values. N=12 for each strain. Greater zone of inhibition corresponds to greater susceptibility to nitrofurantoin. The strains differed significantly in susceptibility.($P= 2.189*10^{-8}$) CWG6555 Δ*(wza-wzb-wzc)* and CWG343 (Δ*wzb*) had significantly larger zone of inhibition than E69 WT while CWG281 (Δ*wza*) had similar sizes of zones of inhibition as the WT. CWG6555 and CWG343 also both had significantly greater zones of inhibition than CWG281 ($P< 0.001$ for both) and zones were larger for CWG6555 than CWG343. ($P<0.001$) Only comparisons between knockouts and the wildtype strain are shown on the plot for clarity. (***:$P<0.001$, "ns"= non significant). **(B)** Representative images of zones of inhibition. Larger halos around the disk correspond to greater susceptibility to nitrofurantoin. Scale bar=10mm. Diameter of zone measured with a ruler. Growth conditions: 18-20 hours at 37°C

K-12 strains was 60µg/ml as no growth was observed in the first 10 hours in the 60µg/ml wells with recovery not occurring until 10-12 hours. Taken together these results give evidence that in liquid media knocking out *wza* in *E. coli* K30 decreases susceptibility to nitrofurantoin while knockouts of the entire cassette or *wzb* do not. Meanwhile no changes in susceptibility are seen when either *wza* and *wzb* are knocked out in *E. coli* K-12.

At 28°C on solid media *wzy* cassette, *wza* and *wzb* knockouts are more susceptible to nitrofurantoin in *E. coli* K30 while in *E. coli* K-12 knocking out *wzb* increases resistance. To examine whether knocking out genes in the *wzy* cassette has the same effect at lower temperatures the disc diffusion assay was conducted at 28°C. Interestingly, as shown in Figure 5, at this lower temperature CWG655 Δ*(wza-wzb-wzc)*, CWG281 *(Δwza)* and CWG343 (Δ*wzb*) were all significantly more susceptible (mean ZOI diameters 24.6, 22.0 and 24.6mm respectively) to nitrofurantoin than the E69 (WT) strain (18.3mm). Additionally, while CWG343 and CWG6555 had similar zones of inhibition, CWG281 had a significantly lower diameter of inhibition than the full cassette knockout CWG655 (Fig. 5). In *E. coli* K-12, JW2047-1 (Δ*wza*) had a smaller diameter of inhibition, demonstrating increased resistance to nitrofurantoin at 28℃ compared to BW25113 (WT) while JW2046-1 (Δ*wzb*) was not significantly different from the WT (Fig. 6). While statistically significant, the magnitude of the change in the mean zone of inhibition between JW2047-1 and BW25113 was only 2mm (Fig. 6B). Mean ZOI diameters for BW25113, JW2047-1 , JW2046-1 were 21.7, 19.7, and 20.8mm respectively. The K30 wildtype strain had significantly smaller zones of inhibition than the K-12 wildtype at 37°C. (bonferroni corrected P=0.017). In conclusion, at 28℃, knocking out genes in the *wzy* cassette changes the phenotype of nitrofurantoin resistance in a differential manner compared to that at 37℃; knocking out both *wza* and *wzb* in *E. coli* K30

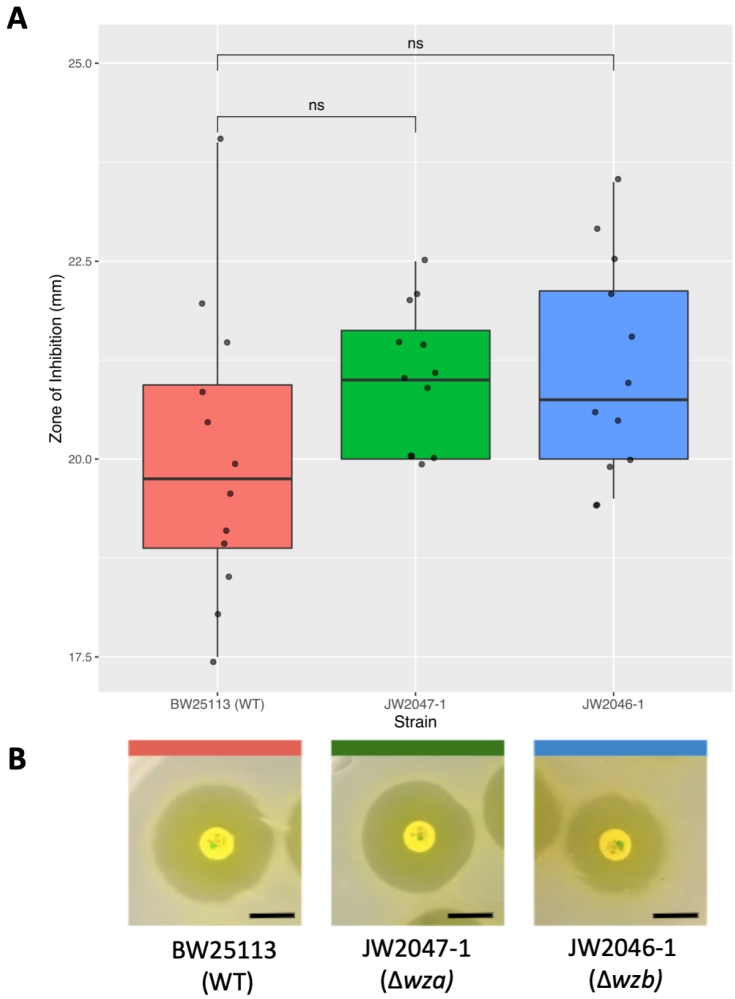

FIG. 3 Deletion of either *wza* or *wzb* in *E. coli* K-12 results in similar susceptibility to nitrofurantoin on solid media at 37°C as determined by disc diffusion assay. (A) Box plot representation of results. N=12 for each strain. Zones of inhibition were not significantly different amongst K-12 BW25113 (WT), JW2047-1 (Δ*wza*), and JW2046-1 (Δ*wzb*) strains. (P= 0.1193) **(B)** Representative images of zones of inhibition. Scale bar=10mm. Growth conditions: 18-20 hours at 37°C

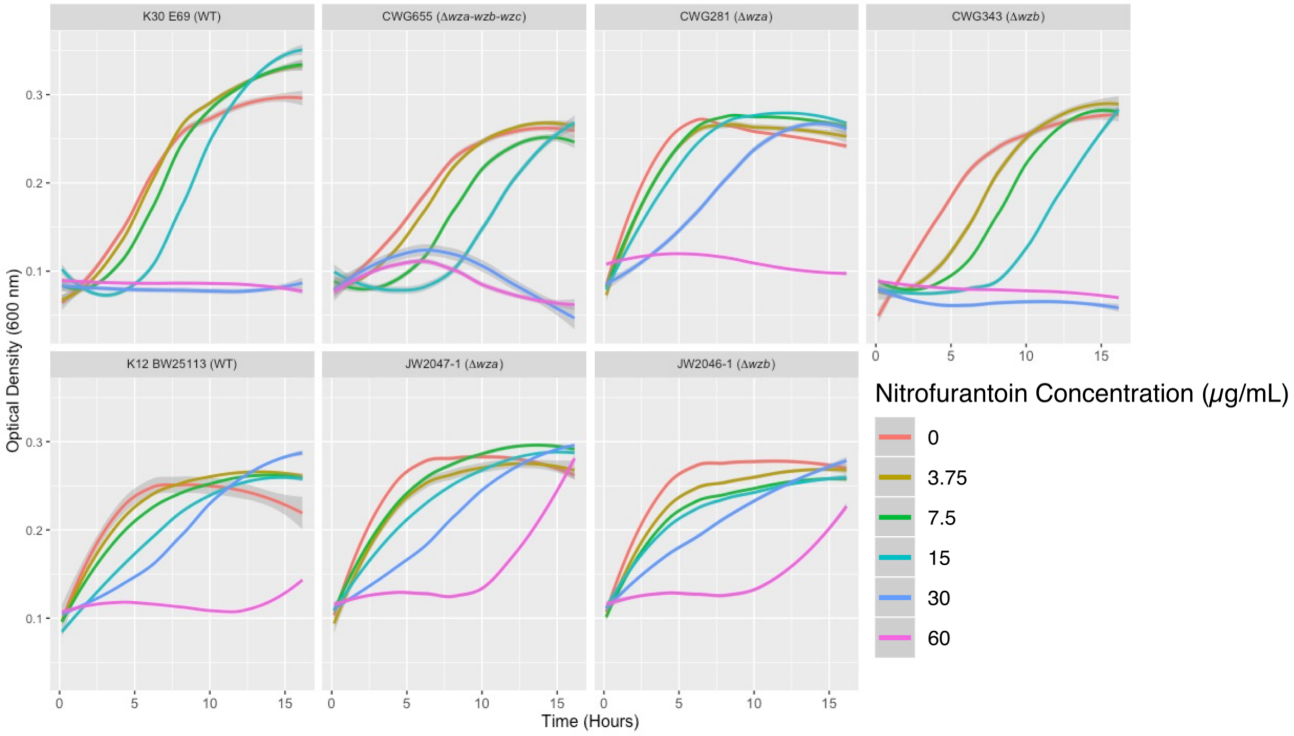

FIG. 4 Growth Curve assay shows a higher minimum inhibitory concentration (MIC) for *wza* knockout in *E. coli* K30 while all *E. coli* K-12 strains have the same MIC. Smoothed growth curves are plotted for each strain in range of stated nitrofurantoin concentrations. OD_{600nm} measurements taken every 10 minutes. Curves representative of duplicate wells for each condition. *E. Coli* K30 strain CWG281 (*Δwza*) grew in nitrofurantoin concentration of 30μg/ml while E69 (WT) and CWG655 Δ*(wza-wzb-wzc)*, and CWG343 (Δ*wzb*) did not. All K-12 strains grew similarly across concentrations. Growth conditions: overnight at 37°C in MH media. OD values for each condition normalized to MH media with corresponding nitrofurantoin concentrations.

is sufficient to increase susceptibility to nitrofurantoin and knocking out *wza* in *E. coli* K-12 slightly increases resistance to nitrofurantoin**.**

Capsule is only present on the *E. coli* K30 wildtype, while all *E. coli* K-12 strains appear acapsular. It has previously been stated that deletion of the *wzy* cassette genes in *E. coli* K30 produces an acapsular phenotype (19). To determine if the knockout strains used in this study exhibited a similar phenotype we selected single colonies that had been grown overnight at either 28 or 37°C and stained them with India ink and visualized the bacteria under a phase contrast microscope. India ink stains the background lightly and the cells dark while any capsule will remain unstained. Clear halos were observed around the *E. coli* K30 E69 (WT), indicating the presence of capsule, whereas no halos were observed around knockout strains CWG6555 Δ*(wza-wzb-wzc),* CWG281 *(Δwza*) and CWG343 (Δ*wzb*), indicating lack of capsule. (Fig. 7A) Neither of the *E. coli* K-12 wildtype or knockout strains had cells with halos at 28°C. (Fig. 7B) Additionally, at 37°C the K-12 BW25113 (WT) was also acapsular (Fig. 7C). This shows that *E. coli* K-12 is acapsular regardless of the presence of the *wzy* cassette components at both 37℃ and 28℃. This experiment confirmed that our knockout strains were phenotypically consistent with the literature.

DISCUSSION

Nitrofurantoin is a commonly prescribed antibiotic for the treatment of UTIs. Previous studies have shown that the *wzy* cassette in *E. coli* K30 plays a role in the resistance to nitrofurantoin and the loss of the *wzb* component increases the susceptibility of *E. coli* K30 to this antibiotic (18, 20) . However, it appears that the *wzb* component may have an opposite effect in *E. coli* K-12, since its deletion led to increased nitrofurantoin resistance (18). Based

on these findings, we hypothesized that deletion of genes within the *wzy* cassette would change the susceptibility to nitrofurantoin differentially in *E. coli* K-12 compared to *E. coli* K30. The findings from all assays testing susceptibility to nitrofurantoin in each of the *wzy* knockout strains are summarized in Table 3.

Our results from the nitrofurantoin disc diffusion assay on *E. coli* K30 strains confirmed what had previously been reported by Gu *et al.*, showing that both the CWG655 full cassette knockout and the CWG343 *wzb* knockout had increased susceptibility to nitrofurantoin, while the CWG281 *wza* knockout had no effect on nitrofurantoin susceptibility (Fig. 2) using disc diffusion assays (18). We propose that in *E. coli* K30 growing on solid media, Wzb itself, or through interactions with Wzc, may be interacting with downstream targets, leading to the deactivation of reductases, promoting nitrofurantoin resistance. Meanwhile, our disc diffusion assays with *E. coli* K-12 showed that the *wzy* cassette components do not have an effect on nitrofurantoin susceptibility, since the JW2047-1 *wza* knockout and JW2046-1 *wzb* knockout had similar zones of inhibition to the wildtype BW25113 (Fig. 3). This is inconsistent with results reported by Gu *et al.*, who also used a disc diffusion assay and found that the JW2046-1 *wzb* knockout actually had slightly increased resistance (18). However, since the difference reported by them was minimal and our results include a higher sample number, we conclude that deletion of *wzb* has no effect on nitrofurantoin susceptibility in *E. coli* K-12 at 37℃. Altogether, these disc diffusion findings indicate that while the *wzy* cassette is influential in nitrofurantoin resistance in *E. coli* K30, it does not play a role in nitrofurantoin resistance in *E. coli* K-12, further highlighting a difference between these two strains.

In order to further elucidate the different roles of the *wzy* cassette in nitrofurantoin resistance between *E. coli* K30 versus *E. coli* K-12 strains, we evaluated the growth of these strains in liquid media in varying concentrations of nitrofurantoin for 16 hours in our MIC assay. Surprisingly, for *E. coli* K30, the results from the MIC assay were different from the disc diffusion assays, showing that knocking out the full cassette or *wzb* has no effect on susceptibility to nitrofurantoin, while the CWG281 *wza* knockout actually had increased

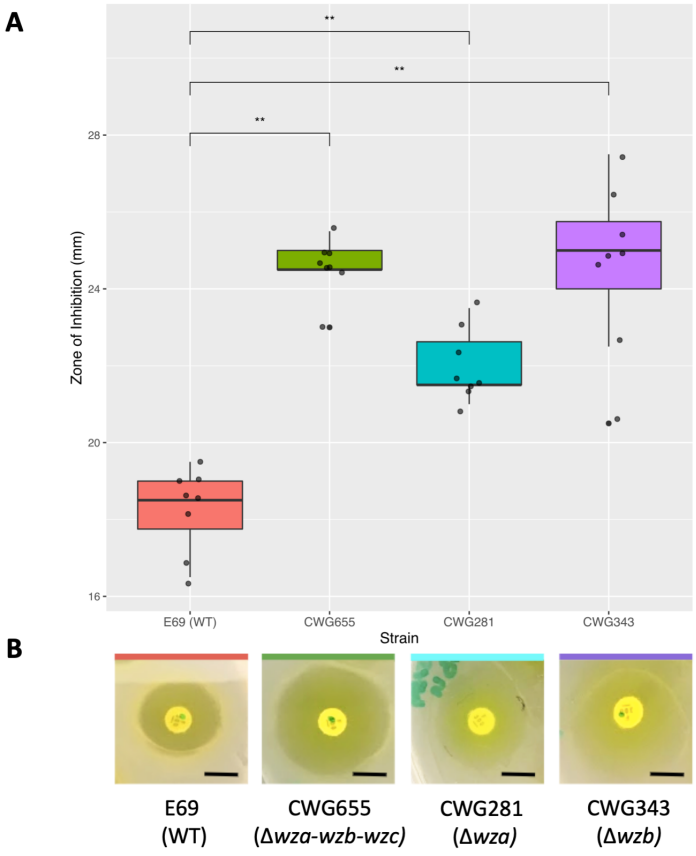

FIG. 5 Deletion of *wza*, *wzb* or full *wzy* cassette results in increased susceptibility to nitrofurantoin in *E. coli* K30 at 28ºC as determined by disc diffusion assay. (A) Box plot representation of results. N=8 for each strain. There was an overall significant change in susceptibility amongst strains.($P= 3.3*10^{-5}$) CWG6555 Δ*(wza-wzb-wzc)*, CWG281 *(Δwza)* and CWG343 (Δ*wzb*) had significantly larger zones of inhibition than E69 WT. CWG655 had significantly greater zones of inhibition than CWG281 (P<0.01). Zones of inhibition were not significantly different between CWG655 and CWG343 and between CWG281and CWG343. Only comparisons between knockouts and the wildtype strain are shown on the plot for clarity (**:P<0.01). **(B)** Representative images of zones of inhibition. Scale bar=10mm. Growth conditions: 18-20 hours at 28ºC

resistance (Fig. 4). The MIC results for *E. coli* K-12 showed similar susceptibility between the WT and *wza* and *wzb* deletion strains, which is consistent with the finding of the disc diffusion assays (Fig. 3).

The observed discrepancies of the knockouts on nitrofurantoin susceptibility between solid and liquid media may be due to a differential surrounding environment. This may involve adaptations in genetic expression, or intracellular signaling within colonies adhered to agar plates, biofilm formation or quorum sensing that may affect gene expression, and consequently antibiotic susceptibility (21-23). Further studies are required to determine why nitrofurantoin susceptibility varies between solid and liquid culture.

Interestingly, while all the *E. coli* K-12 strains had a similar final OD_{600} for all concentrations of nitrofurantoin, the growth curves showed that their growth was delayed until about 10 hours at nitrofurantoin concentrations of 60µg/ml (Fig. 4). Nitrofurantoin is a bacteriostatic antibiotic at low concentrations, but it is bactericidal at high concentrations, thus leading to death of the *E. coli* (5, 24). However, in liquid cultures, a population of survivor bacteria, known as persistors, may be able to evade the antibiotic killing (25). These remaining bacteria may stay at a constant level until they reach mid-exponential phase, when they start growing and dividing rapidly (25). The time point when growth of the 60µg/ml nitrofurantoin cultures begins coincides with the mid-exponential phase of the other *E. coli* K-12 cultures (Fig. 4), suggesting that this delayed growth seen in the 60µg/ml nitrofurantoin cultures is due to the presence of this persistor population.

While all these tests were done at 37℃, the optimal growth temperature for *E. coli*, colanic acid production by *E. coli* K-12 occurs under conditions of stress or at temperatures under 30℃ (26). Considering the *wzy* cassette is responsible for colanic acid production rather than Type I CPS production in *E. coli* K-12, we speculated that we were not seeing differences between K-12 *wzy* cassette knockouts and the wildtype because the *wzy* cassette was not being

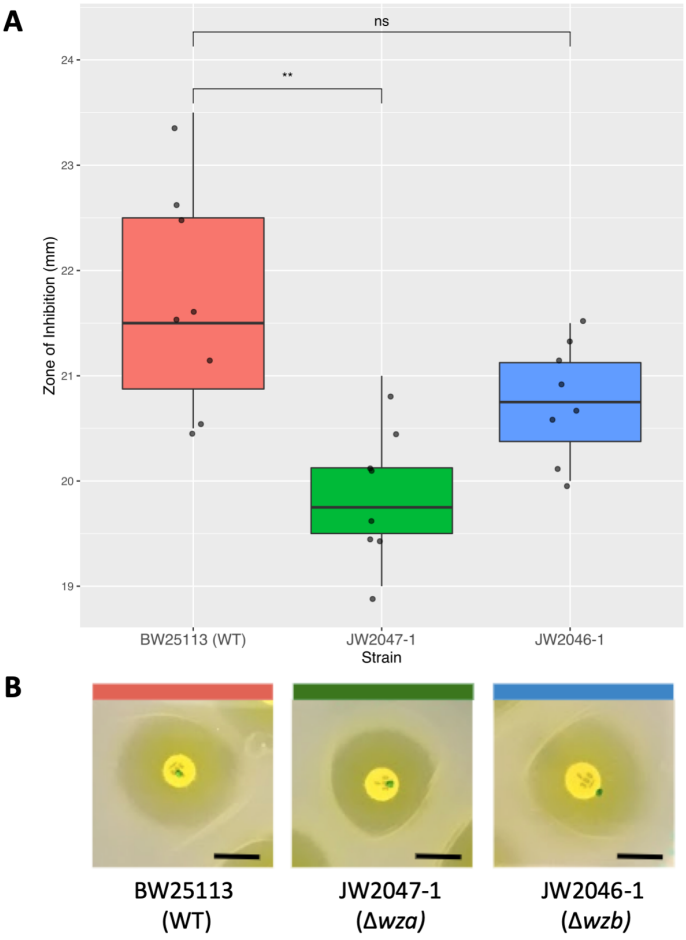

FIG. 6 Deletion of *wza* results in increased resistance to nitrofurantoin in *E. coli* K-12 at 28ºC as determined by disc diffusion assay. (A) Box plot representation of results. N=8 for each strain. There was an overall significant change in susceptibility amongst strains.(P= 0.002608). Zones of inhibition were significantly smaller in the JW2047-1 (Δ*wza*) strain compared to the K-12 BW25113 (WT) strain. No significant difference observed between BW25113 and JW2046-1 (Δ*wzb*) or between JW2047-1 and JW2046-1. Only comparisons between knockouts and the wildtype strain are shown on the plot for clarity.(**= P<0.01, "ns"= non-significant.) **(B)** Representative images of zones of inhibition. Scale bar=10mm. Growth conditions: 18-20 hours at 28ºC

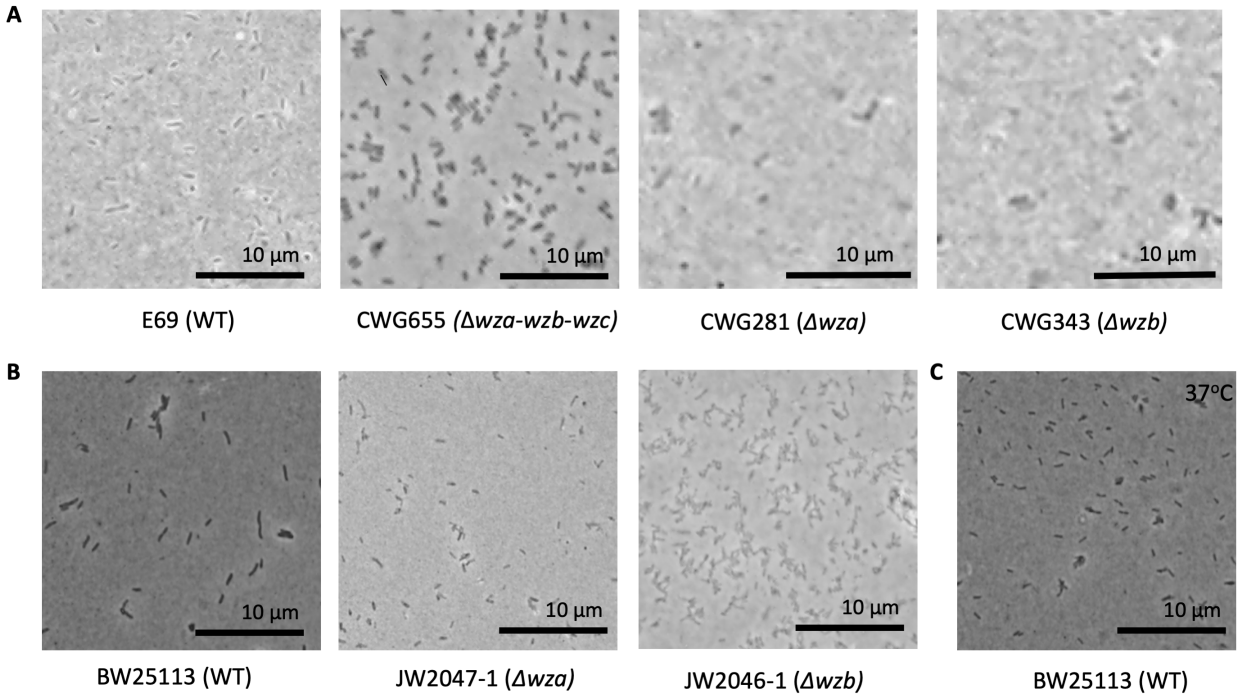

FIG. 7 Presence of capsule observed in *E. coli* K30 wildtype but not in *wzy* knockouts or K-12 wildtype and knockouts by India ink staining. A) Micrograph of *E. coli* K30 wildtype and knockout strains stained with India ink. Cells are darkly stained with capsule appearing as a white, unstained halo around the cell on a stained background. E69 (WT) appears capsular while CWG6555 Δ*(wza-wzb-wzc)*, CWG281 *(Δwza)* and CWG343 (Δ*wzb*) appear acapsular. Growth conditions: 28ºC overnight on MH agar plates. Single colonies selected for staining. Scale bar=10μM. Images converted to greyscale post capture. **B)** K-12 wildtype and knockouts are all acapsular. Growth conditions 28ºC overnight on MH agar plates **C)** K-12 BW25113 (WT) is acaspular at 37ºC.

expressed. Therefore, disc diffusion assays were performed at 28℃ to induce expression of the *wzy* cassette components in *E. coli* K-12. For *E. coli* K30, deletion of the full cassette and *wzb* again resulted in increased susceptibility as it did at 37℃, however deletion of *wza* also lead to increased nitrofurantoin susceptibility compared to the wildtype even though at 37℃, *wza* had no effect (Fig. 5). This suggests that in conditions of stress, such as lower temperatures, both Wza and Wzb of the *wzy* cassette may be important in the mechanism to nitrofurantoin resistance in *E. coli* K30. At 28℃, differences were seen between the K-12 strains, as deletion of *wza* lead to increased resistance (Fig. 6), suggesting that the cassette is being expressed and may have a function in nitrofurantoin susceptibility. However, since only deletion of *wza* seems to affect nitrofurantoin susceptibility in *E. coli* K-12 it is possible that its resistance mechanism is different than the one observed in *E. coli* K30. Wza has been found to promote bacterial susceptibility to some antibiotics by possibly acting as a portal of entry (27). The fact that deletion of *wza* results in increased resistance supports a hypothesis that in *E. coli* K-12, at temperatures where the *wzy* cassette is expressed, nitrofurantoin may be using Wza as a portal of entry into the bacteria.

Polysaccharide capsule produced by gram negative bacteria has been shown to provide a mechanism of resistance to antibacterial proteins and peptides, likely through limiting their interaction with the bacterial surface and therefore their penetration into the bacteria (25, 26). We decided to investigate if the presence of capsule could explain the differential susceptibilities to nitrofurantoin observed since the *wzy* cassette is involved in capsule production and colanic acid production in *E. coli* K30 and *E. coli* K-12, respectively (7, 8). India ink staining allowed for visualization of capsule in the *E. coli* K30 WT, however not in any *wzy* cassette knockout strains in *E. coli* K30 (Fig. 7A). The *E. coli* K-12 strains all lacked visible capsule (Fig. 7B-C), which is consistent with data that *E. coli* K-12 is acapsular and expresses low amounts of colanic acid at 37℃ (17). The absence of capsule in CWG281 *wza*

knockout would suggest an increased susceptibility if capsule was promoting resistance to nitrofurantoin. However, the disc diffusion assay and MIC assay show deletion of *wza* in *E. coli* K30 results in no difference in susceptibility or increased resistance. This suggests that the nitrofurantoin resistance mechanism is capsule independent, since lack of capsule does not always infer increased susceptibility.

It should be noted that the wildtype strains *E. coli* K30 and *E. coli* K-12 had a statistically significant difference in their susceptibility to nitrofurantoin at both 37℃ and 28℃. At 37℃ the *E. coli* K30 wildtype had an increased susceptibility to nitrofurantoin compared to the *E. coli* K-12 wildtype. While at 28℃ the *E. coli* K30 wildtype was actually more resistant to nitrofurantoin compared to the *E. coli* K-12 wildtype. It is important to note that the difference in susceptibility between wildtype strains was not part of our original question and the comparison was made after the original analysis, a practice that can increase the chance of capturing a spurious relationship. However, this raises questions about other pathways that differentially contribute to nitrofurantoin resistance in the wildtypes and possibly interactions between those pathways and *wzy* cassette products are responsible for the differing results seen in the knockout strains at 37℃ and 28℃.

While the differences observed in the different *E. coli* K30 strains can be attributed to the different roles of the *wzy* cassette components, the difference in the role of the cassette in nitrofurantoin resistance in the *E. coli* K30 compared to *E. coli* K-12 may be explained by regulatory differences. The *wzy* cassette is regulated by the Regulator of capsule synthesis (Rcs) two component regulator system, consisting of RcsA, RcsB, and RcsC (17). These genes are highly conserved between *E. coli* K-12 and K30, however the regions upstream of the *cps* gene cluster, which includes the *wzy* cassette genes, is different between these strains (27). Additionally, deletion of *rscA* or *rscB* impairs colanic acid production in *E. coli* K-12 but does not affect Type 1 CPS production in *E. coli* K30, indicating another uncharacterized regulatory mechanism may exist for CPS production in *E. coli* K30 (27). It also remains unclear whether RcsC, the transmembrane sensor of this regulatory system, responds to the same stimuli in *E. coli* K30 versus K-12 (17). Differential sensing through RcsC or regulation through an unknown mechanism in *E. coli* K30 may have led to differential expression of the *wza, wzb,* and *wzc* compared to *E. coli* K-12, which may have accounted for the different roles of the *wzy* cassette components between the two strains.

Limitations Because of time and equipment restrictions there are several methodological limitations to this study. To confirm the genotypes of the strains used we were only able to do so via PCR and gel electrophoresis. To increase our confidence, especially in cases where the WT and knockout products were closer in size, we would have liked to sequence the amplified products. Due to time concerns we were not able to repeat the India Ink assays to obtain better quality images. Better resolution microscopy would have given a clearer indication of capsule presence. While we obtained consistent results from our MIC assays, only two runs were performed. It would be beneficial to perform more MIC assays with different ranges of nitrofurantoin concentrations to strengthen our results and to more specifically determine the minimum inhibitory concentration for each strain.

Conclusions In conclusion, our study shows a differential role of the *wzy* cassette in nitrofurantoin resistance in *E. coli* K30 versus *E. coli* K-12. Our findings indicate a role of the *wzy* cassette in nitrofurantoin resistance in *E. coli* K30. However, considering deletions of *wza* and *wzb* had no effect on nitrofurantoin resistance in *E. coli* K-12 at 37℃, we conclude that these *wzy* cassette components do not play a role in nitrofurantoin resistance at this temperature but may at temperatures below 30°C. Furthermore, our data on capsule production points towards a nitrofurantoin resistance mechanism that is capsule independent.

Future Directions The role of *wzc* in nitrofurantoin resistance still remains unknown in both K30 and K-12 *E. coli* strains. Wzc is essential for the production of capsule in *E. coli* K30 and colanic acid in *E. coli* K-12. Due to its importance in these mechanisms, it would be interesting to repeat our disc diffusion assays and MIC assays with a *wzc* knockout for both K30 and K-12 *E. coli* strains. Additionally, since the phosphorylation activity of Wzc is essential for its function in capsule production, specifically mutating Wzc's C-terminal Y-

cluster so it cannot autophosphorylate could provide insight into if this phosphorylation activity is what is contributing the nitrofurantoin resistance mechanism.

The differences in nitrofurantoin resistance of the *wzy* cassette knockouts in *E. coli* K30 versus K-12 strains may be due to differences in regulation of the cassette within these two strains. Additionally, it is thought that *E. coli* K-12 only expresses these genes to make colanic acid under stress or at temperatures under 30℃ (17, 26). Higher colanic acid gene expression may be induced in *E. coli* K-12 if minimal media is used, or if the temperature is further reduced (17). Therefore, it would be worthwhile to determine the expression levels of Wza, Wzb, and Wzc within these two strains at both 37℃ and 28℃ to see if they are differentially expressed and to see if they are expressed in *E. coli* K-12 at either of these temperatures, or under more stressful conditions. Reverse-transcriptase qPCR analysis should be done to evaluate mRNA levels of each of the cassette components within these two strains to compare their expression levels, and see if this could explain the differences in nitrofurantoin resistance between *E. coli* K-12 and *E. coli* K30 strains. Additionally, it would be interesting to test if there is differential gene expression in *E. coli* K30 strains grown on solid versus liquid media through qPCR of the *wzy* cassette components to see if this could explain the conflicting results seen in the disc diffusion assay versus the MIC assay. Since the Rcs two-component system has differing upstream regions in *E. coli* K-12 and K30 which control *rcs* gene regulation, it would also be worthwhile to determine if there are differing levels of these capsule-regulating genes in each cell to see if this system is involved in differential capsule production under different conditions, such as temperature or liquid versus solid media, which may be determined via reverse-transcriptase qPCR (27).

In order to further elucidate if Wzb plays a role in nitrofurantoin resistance, it would be beneficial to determine other targets of this phosphatase in order to assemble a potential resistance mechanism. Presently, the targets of Wzb and similar phosphotyrosine phosphatases are not well characterized (28). It is suspected that Wzb may have other unidentified substrate targets, and these targets may be involved in the differential resistance to nitrofurantoin, though more studies are required to determine if there is a relationship (28, 29).

ACKNOWLEDGEMENTS

This project was funded by the Department of Microbiology and Immunology at the University of British Columbia. We would like to thank Dr. Chris Whitfield at the University of Guelph for providing us with the *E. coli* K30 mutants and the Department of Microbiology for providing us with the K-12 strains and materials. Thank you to Dr. David Oliver, Reynold Farrera, Dr. Evelyn Sun and Craig Kornak for support, guidance and materials. Furthermore, we would like to thank the media room staff for handling material autoclaving and disposal. We would like to thank the UBC BioImaging facility, particularly Miki Fujita for microscope booking and Kevin Hodgeson for providing information and aid with sample preparation and phase contrast microscope hardware and software. Finally, thank you to Linda Horianopoulos for providing our team with India ink, consulting our staining methods and aiding us with data interpretation. We would also like to thank two anonymous reviewers for constructive feedback on this manuscript.

CONTRIBUTIONS

RT: Derived research question and initial experimental design. Wrote the title, abstract, introduction, acknowledgements and a portion of methods and materials and results. Edited the results, discussion, future direction sections and overall paper edits. Setup PCR resulting in figure S1, disc diffusion assays resulting in figures 2, 3, 5, and 6, and assisted in MIC assay resulting in figures 4 and S2. Prepared slides and preformed imaging in India ink assay in figure 7.

GS: Contributed to the refinement of the research question and experimental design. Wrote the methods and materials, discussion, conclusion, and future directions. Edited the abstract, introduction and results sections and overall paper edits. Ran electrophoresis resulting in figure S1, disc diffusion assays resulting in figures 2, 3, 5, and 6, and assisted in MIC assay resulting in figures 4 and S2. Prepared slides for imaging in India ink assay in figure 7.

MS: Contributed to the refinement of research question and experimental design. Wrote the results section, portion of the methods and materials, and performed statistical analyses and made figures. Edited the abstract, introduction, methods, and discussion and future discussion sections and overall paper edits.

Ran electrophoresis resulting in figure S1B, disc diffusion assays resulting in figures 2, 3, 5, and 6, and MIC assay resulting in figures 4 and S2.

REFERENCES

1. **Flores-Mireles AL, Walker JN, Caparon M, Hultgren SJ.** 2015. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nature Reviews Microbiology. **13:**269-284.
2. **Foxman B.** 2010. The epidemiology of urinary tract infection. Nature Reviews Urology. **7:**653.
3. **Huttner A, Verhaegh EM, Harbarth S, Muller AE, Theuretzbacher U, Mouton JW.** 2015. Nitrofurantoin revisited: a systematic review and meta-analysis of controlled trials. J. Antimicrob. Chemother. **70:**2456-2464.
4. **McOsker CC, Fitzpatrick PM.** 1994. Nitrofurantoin: mechanism of action and implications for resistance development in common uropathogens. J. Antimicrob. Chemother. **33:**23-30.
5. **Sandegren L, Lindqvist A, Kahlmeter G, Andersson DI.** 2008. Nitrofurantoin resistance mechanism and fitness cost in *Escherichia coli.* J. Antimicrob. Chemother. **62:**495-503.
6. **Sachdeva S, Palur RV, Sudhakar KU, Rathinavelan T.** 2017. *E. coli* group 1 capsular polysaccharide exportation nanomachinary as a plausible antivirulence target in the perspective of emerging antimicrobial resistance. Frontiers in Microbiology. **8:**70.
7. **Whitfield C, Paiment A.** 2003. Biosynthesis and assembly of Group 1 capsular polysaccharides in *Escherichia coli* and related extracellular polysaccharides in other bacteria. Carbohydr. Res. **338:**2491-2502.
8. **Whitfield C.** 2006. Biosynthesis and Assembly of Capsular Polysaccharides in *Escherichia coli*. Annu. Rev. Biochem. **75:**39-68. doi: 10.1146/annurev.biochem.75.103004.142545. https://doi.org/10.1146/annurev.biochem.75.103004.142545.
9. **Reid AN, Whitfield C.** 2005. Functional analysis of conserved gene products involved in assembly of *Escherichia coli* capsules and exopolysaccharides: evidence for molecular recognition between Wza and Wzc for colanic acid biosynthesis. J. Bacteriol. **187:**5470-5481.
10. **Patel KB, Furlong SE, Valvano MA.** 2010. Functional analysis of the C-terminal domain of the WbaP protein that mediates initiation of O antigen synthesis in *Salmonella enterica*. Glycobiology. **20:**1389-1401.
11. **Paiment A, Hocking J, Whitfield C.** 2002. Impact of phosphorylation of specific residues in the tyrosine autokinase, Wzc, on its activity in assembly of group 1 capsules in *Escherichia coli*. J. Bacteriol. **184:**6437-6447.
12. **Wugeditsch T, Paiment A, Hocking J, Drummelsmith J, Forrester C, Whitfield C.** 2001. Phosphorylation of Wzc, a tyrosine autokinase, is essential for assembly of group 1 capsular polysaccharides in *Escherichia coli*. J. Biol. Chem. **276:**2361-2371.
13. **Vincent C, Duclos B, Grangeasse C, Vaganay E, Riberty M, Cozzone AJ, Doublet P.** 2000. Relationship between exopolysaccharide production and protein-tyrosine phosphorylation in gram-negative bacteria. J. Mol. Biol. **304:**311-321.
14. **Beis K, Collins RF, Ford RC, Kamis AB, Whitfield C, Naismith JH.** 2004. Three-dimensional structure of Wza, the protein required for translocation of group 1 capsular polysaccharide across the outer membrane of *Escherichia coli*. J. Biol. Chem. **279:**28227-28232.
15. **Bushell S, Mainprize I, Wear M, Lou H, Whitfield C, Naismith J.** 2013. Wzi Is an Outer Membrane Lectin that Underpins Group 1 Capsule Assembly in *Escherichia coli.* Structure. **21:**844-853. doi: 10.1016/j.str.2013.03.010. https://www.sciencedirect.com/science/article/pii/S0969212613001081.
16. **Ranjit DK, Young KD.** 2016. Colanic acid intermediates prevent de novo shape recovery of *Escherichia coli* spheroplasts, calling into question biological roles previously attributed to colanic acid. J. Bacteriol. **198:**1230-1240.
17. **Whitfield C, Roberts IS.** 1999. Structure, assembly and regulation of expression of capsules in *Escherichia coli.* Mol. Microbiol. **31:**1307-1319.
18. **Gu M, Khan A, Pagulayan DS, Tam WL.** 2018. Deletion of the capsule phosphatase gene *wzb* renders *Escherichia coli* strain K30 sensitive to the antibiotic nitrofurantoin. J.Exp.Microbiol.Immunol. **4:**1-15.
19. **Reid AN, Whitfield C.** 2005. functional analysis of conserved gene products involved in assembly of *Escherichia coli* capsules and exopolysaccharides: evidence for molecular recognition between Wza and Wzc for colanic acid biosynthesis. J. Bacteriol. **187:**5470-5481. doi: 187/15/5470-a [pii].
20. **Botros S, Mitchell D, Van Ommen C.** 2015. Deletion of the *Escherichia coli* K30 group I capsule biosynthesis genes *wza, wzb* and *wzc* confers capsule-independent resistance to macrolide antibiotics. J.Exp.Microbiol.Immunol. **19:**1-8.
21. **Fujikawa H, Morozumi S.** 2005. Modeling surface growth of *Escherichia coli* on agar plates. Appl.Environ.Microbiol. **71:**7920-7926.
22. **Melke P, Sahlin P, Levchenko A, Jönsson H.** 2010. A cell-based model for quorum sensing in heterogeneous bacterial colonies. PLoS Computational Biology. **6:**e1000819.
23. **Jeanson S, Floury J, Gagnaire V, Lortal S, Thierry A.** 2015. Bacterial colonies in solid media and foods: a review on their growth and interactions with the micro-environment. Frontiers in Microbiology. **6:**1284.

24. **Kashanian J, Hakimian P, Blute Jr M, Wong J, Khanna H, Wise G, Shabsigh R.** 2008. Nitrofurantoin: the return of an old friend in the wake of growing resistance. BJU Int. **102:**1634-1637.
25. **Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K.** 2004. Persister cells and tolerance to antimicrobials. FEMS Microbiol. Lett. **230:**13-18.
26. **Whitfield C, Paiment A.** 2003. Biosynthesis and assembly of Group 1 capsular polysaccharides in *Escherichia coli* and related extracellular polysaccharides in other bacteria. Carbohydr. Res. **338:**2491-2502.
27. **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. Vol. **21:**108-112.
28. **Grangeasse C, Nessler S, Mijakovic I.** 2012. Bacterial tyrosine kinases: evolution, biological function and structural insights. Philosophical Transactions of the Royal Society B: Biological Sciences. **367:**2640-2655.
29. **Lescop E, Hu Y, Xu H, Hu W, Chen J, Xia B, Jin C.** 2006. The solution structure of *Escherichia coli* Wzb reveals a novel substrate recognition mechanism of prokaryotic low molecular weight protein-tyrosine phosphatases. J. Biol. Chem. **281:**19570-19577