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Investigation of outer membrane porin OmpC mutation mediated relationship between Cefotaxime antibiotic resistance and T4 phage resistance in *Escherichia coli*

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SUMMARY Outer membrane porin C (OmpC) is a protein porin found on the outer membrane of Gram-negative bacteria. In *Escherichia coli,* OmpC allows entry of hydrophilic molecules in to the cell, including cefotaxime (CTX), a cephalosporin antibiotic. Previous research has shown that clinical isolates of multidrug resistant *E. coli* with mutations in OmpC have increased resistance to cefotaxime. Research has also shown OmpC as a necessary receptor for T4 bacteriophage entry. Due to their mutual dependence on OmpC for entry, we hypothesize that exposure to cefotaxime would select for OmpC mutations that also confer resistance to T4 phage in *E. coli* BW25113. To test this, we first used a minimum inhibitory concentration (MIC) assay to confirm that deletion of *ompC* increased *E. coli* resistance to cefotaxime. With validation of this phenotypic difference, we selected for strains that showed comparable antibiotic resistance to the knockout via sequential passaging of *E. coli* BW25113 in increasing cefotaxime concentrations. When we subjected these resistant cultures to T4 phage, we saw trends suggesting decreased and delayed lysis as predicted. To confirm that OmpC was indeed the target of selection, we sequenced the *ompC* gene from these double resistant isolates and found consistent insertion mutations in a number of the isolates. The insertion was predicted to encode an α-helical motif protruding into the channel. This paper addresses the relationship between antibiotics and bacteriophage, an under-researched area in clinical microbiology, and holds implications for how we approach antibiotic therapy and multidrug resistant bacteria.

INTRODUCTION

OmpC is an outer membrane porin protein expressed by Gram-negative bacteria, including *Escherichia coli* (1) The porin structure is trimeric and consists of 16-stranded beta barrels with negatively-charged amino acids lining the extracellular L3 loop and positively charged amino acids on the opposite barrel wall. These regions form the constriction zone of the porin and contribute to its size-exclusion and permeability properties (2, 3). OmpC has previously been described in facilitating nutrient uptake and antibiotic influx and is exploited as an attachment receptor to *E. coli* by T4 bacteriophage (1, 4, 5). OmpC is preferentially expressed under high osmolarity conditions over OmpF, another porin channel (5). Due to the negative charges within OmpC, compounds with multiple negative charges penetrate slowly, while zwitterionic molecules or molecules with only one negative charge penetrate more rapidly (6).

Cefotaxime is an antibiotic of the cephalosporin class of β-lactam antibiotics that is effective against Gram-positive and Gram-negative bacteria and has a physiological charge of negative one. Resistance to cefotaxime can result from β-lactamase production, modification of its target penicillin-binding protein or altered membrane permeability (7). *E. coli* has been shown to develop resistance to cephalosporins by decreased OmpC and OmpF porin expression, as they cannot induce chromosomal beta-lactamase expression in response to cephalosporins (3, 8). Lou et al. have shown that mutations altering the electrostatic field established within the constriction zone of OmpC in multidrug resistant *E. coli* can disrupt antibiotic-porin interactions. Cefotaxime efficacy was affected by these types of mutations, conferring resistance. They confirmed OmpC's role in cefotaxime resistance by complementation with wildtype (WT) *E. coli* K12 OmpC in these clinical samples; effectively lowering the minimum inhibitory concentration (MIC) (9).

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Additionally, bacteria with high resistance to cephalosporin antibiotics have been found to have mutations in the L3 loop of porins homologous to OmpC (4). We predicted that by selecting for cefotaxime resistance in *E. coli* we would observe mutations in *ompC* in resistant strains compared to *ompC* in WT *E. coli* BW25113.

Previous studies have shown evidence supporting the existence of a relationship between antibiotic and phage susceptibility. Kim et al. have demonstrated synergistic bactericidal effects by combining antibiotic and phage treatment (10). Chan et al. showed that mutations selected for phage resistance increased antibiotic susceptibility in *Pseudomonas aeruginosa* (11). However, the question of whether the development of antibiotic resistance can also lead to phage resistance has not been explored. We predicted that mutations in *ompC* that contribute to cefotaxime resistance also contribute to delayed phage lysis because both cefotaxime and T4 phage use OmpC for entry into the cell and our selection process would select for mutations that confer resistance to both cefotaxime and T4 phage.

METHODS AND MATERIALS

Bacterial strains and culture conditions. *E. coli* strains BW25113 (wild-type), JW2203-1 (*ΔompC*), and DH5α (containing pUC19) were obtained from the Ramey Database from the Microbiology and Immunology Department at the University of British Columbia. Media used for cell growth include Luria Bertani (LB) medium, L medium, M9 (+20% sucrose) medium (M9S), and M9S medium supplemented with cefotaxime at various concentrations. See supplemental materials for media recipes. All strains used are summarized in Table 1.

Preparation of antibiotic stock solutions. Cefotaxime (200 mg/mL) stock solution was prepared by dissolving Cefotaxime sodium salt (Alfa Aesar) or ampicillin sodium salt (Bio Basic) in distilled water, and then filter sterilizing through a 0.22 μm filter (VWR). Stock solutions were stored at -20°C.

Confirmation of *ompC* knockout from *E. coli* JW2203-1. Colony lysis PCR was performed using primers flanking the *ompC* gene (Table 2). *E. coli* BW25113 (wild-type) and JW2203-1 (*ΔompC*) were streaked on LB agar plates and incubated at 37°C overnight. The next day, one colony of each strain was suspended in 1 mL of sterile dH_2O and lysed in 95 ºC water bath for 10 mins. The tubes were centrifuged at 8000 RPM for 10 mins and the supernatant (containing the genomic DNA) was transformed to a clean tube. pUC19 (obtained from MICB 421 collection) was used as a PCR positive control. The PCR reaction was set up by the method of Chang et al. with the Bio-Rad T100 Thermal Cycler using Platinum Taq DNA polymerase and buffer (Invitrogen) (1). See Table 3 for the PCR conditions. The PCR products and a 1 kb DNA ladder (Invitrogen) were electrophoresed on

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

Name of Strain	Description	Experiments
BW25113	Wild-type	• T4 phage propagation and confirmation of purity • Minimum inhibitory concentration assay to determine cefotaxime susceptibility • Selection for T4 Phage resistance • Sequencing of *ompC*
JW2203-1	*ΔompC::kan*	• Minimum inhibitory concentration assay to determine cefotaxime susceptibility • Selection for T4 Phage resistance

TABLE. 2 PCR primer sets

Gene	Primer Sequence (5'-3')
T4 *gp23*	Forward: AGAATCGGTCCAGTGCGTTT
	Reverse: GATTGACTGGTGGGGAGTGG
pUC19	Forward: GTGAAATACCGCACAGATGC
	Reverse: GGCGTTACCCAACTTAATCG
ompC	Forward: GCAGGCCCTTTGTTCGATATCAATC
	Reverse: ATCAGTATGCAGTGGCATAAAAAAGC

a 1% (w/v) agarose gel containing SYBR Safe DNA Stain in 0.5X TBE (Invitrogen) at 110V in 0.5X TBE. The gel was then visualized under UV light with the Alpha Innotech MultiImage light cabinet

T4 phage propagation and confirmation of purity. Bacteriophage T4 was obtained from the Microbiology and Immunology Department at the University of British Columbia. T4 phage was added to a log-phase *E. coli* BW25113 culture grown in L medium at MOI of 0.1 and incubated (200 RPM, 37°C) for 4 hours until the cells lysed completely. The lysed culture was then centrifuged at 8000 RCF for 10 mins, and the supernatant (containing the phages) was filter sterilized through a 0.22 µm filter (VWR) and stored at 4°C. A double agar overlay plaque assay was conducted to determine phage titer. Serial dilution by 10-fold factor up to 10^8 of the phage stocks was performed. 100 µL of each dilution was added to 3 mL of molten agar (~50ºC) along with 30 µL of *E. coli* BW25113 log-phase culture, vortexed, and quickly poured on top of LB agar, and then incubated at 37°C overnight. Phage titer was determined by the following calculation:

Titre = (Number of plaques) x (10) x (Dilution factor)

TABLE. 3 PCR settings

Gene to be amplified	PCR Step	Cycles	Temperature (℃)	Time (min)
T4 *gp23*	Initial Denaturation	1	93	10
	Denaturation	40	93	1
	Annealing	40	52	1
	Extension	40	72	1
	Final Extension	1	72	5
ompC	Initial Denaturation	1	94	2
	Denaturation	35	94	0.5
	Annealing	35	52.5	0.5
	Extension	35	72	1.5
	Final Extension	1	72	1

To confirm the purity of the working stock, PCR amplification and gel electrophoresis were performed with primers flanking T4 *gp23*. pUC19 was used as a PCR positive control. The PCR reaction was carried out by the method of Chan et al. with the Bio-Rad T100 Thermal Cycler using Platinum Taq DNA polymerase and buffer (Invitrogen) (12). PCR conditions and primers are outlined in Tables 2 and 3. The PCR products and a 1 kb DNA ladder (Invitrogen) were electrophoresed on a 1% (w/v) agarose gel containing SYBR Safe DNA Stain in 0.5X TBE (Invitrogen) at 110V in 1X TBE. The gel was then visualized under UV light with the Alpha Innotech MultiImage light cabinet.

Minimum inhibitory concentration assay to determine cefotaxime susceptibility. Cefotaxime was serially dilution by a factor of 2 starting from 20 µg/mL to 0.15 µg/mL using M9S medium. 200 µL of each concentration was added to a 96 wells plates along with control wells contained M9S medium without cefotaxime. Log-phase culture of the tested strain was diluted down to an OD600 of 0.1, and 10 µL was added to each well. The plate was then incubated at 37°C until proper growth in the control wells was observed (between 1 - 4 days) before reading the OD600 on the BioTek Epoch Microplate Spectrophotometer. Minimum inhibitory concentration (MIC) was determined as the lowest concentration of cefotaxime that inhibited the growth of bacteria from reaching an OD600 of 0.1 after 4 days.

Selection for resistance to cefotaxime. Sequential passaging of *E. coli* BW25113 with increasing concentration of cefotaxime was performed to select for resistant cultures. Concentration gradients increasing by 0.05 µg/mL of cefotaxime was made with M9S media starting with the determined MIC concentration for the wild-type *E. coli* BW25113. 200 µL of this gradient concentration was aliquoted in 96 well plates along with positive control wells containing M9S medium with no antibiotic and negative control wells with increasing antibiotic but no inoculum. 10 µL of log-phase *E. coli* BW25113 culture obtained from the highest concentration wells from the MIC test was used to inoculate appropriate wells. The plate was incubated and monitored daily up to day 4 for growth in the increased concentrations by reading the OD600 on the BioTek Epoch Microplate Spectrophotometer. Sufficient growth was determined when wells reached an OD600 >0.1. Once growth was observed, 10 µL of the culture in wells containing the highest concentration of cefotaxime was used to inoculate another gradient 96 wells plate. The new plate then starts at 0.1 µg/mL CTX below the observed MIC and increases again by 0.05 µg/mL. This passaging was repeated 5 times until selected strains were resistant at 1.35 µg/mL CTX which were then named cefotaxime resistant (CR) cultures. The cultures were

TABLE. 4 Selection of cefotaxime resistant BW25113

Passage #	Days	Conc. CTX (µg/mL)
Initial MIC	0-1	0.3125
1	1-2	0.45
2	2-3	0.55
3	3-6	0.75
4	6-10	1.15
5	10-14	1.35
Final MIC	15-19	2.5

then passaged into LB broth, allowed to grow overnight and a new MIC was conducted by same methods as previously described.

Selection for T4 Phage resistance in cefotaxime resistant cultures. Cefotaxime resistant cultures (CR 1 and 2), *E. coli* BW25113 (WT) and JW2203-1 (*ΔompC*) were grown in LB overnight. The next day, overnight cultures were seeded into 96 wells plates at OD600 = 0.2 in 200 µL LB. Lysis conditions were performed in triplicates and a no phage control was included for each strain. The plate was incubated at 37°C for 3 hours until an OD600 of approximately 0.4 was reached. At Time = 3 h, T4 phage was added to each well at an MOI of 0.1 calculated based on the individual OD600 readings of each well. The plate was incubated and read on the BioTek Epoch Microplate Spectrophotometer every 30 mins for 8 hours total. The plate was then incubated for 48 hours at 37°C. After 48 hours, T4 phage infected wells that contained OD600 > 0.3 cultures were assumed to be resistant to T4 and grew post lysis. Inoculums from these cultures were used to streak M9S agar plates and isolate pure colonies of "double resistant isolates". Another MIC assay for cefotaxime was performed on these isolates to confirm double resistance.

TABLE. 5 MIC of DAE219 N1-20. *DAE219 N5 and N20 sequences were not available. Cut off for growth at OD600> 0.1

Isolate code	Mutation (+/-)	Highest [CTX] tolerated (µg/mL)
DAE219 N1-4	+	1.25
DAE219 N5	N/A*	5.0
DAE219 N6	+	1.25
DAE219 N7	+	5.0
DAE219 N8	+	5.0
DAE219 N9	+	1.25
DAE219 N10	+	5.0
DAE219 N11	+	0.625
DAE219 N12	-	1.25
DAE219 N13	-	0.625
DAE219 N14	-	0.625
DAE219 N15	-	0.625
DAE219 N16	-	0.625
DAE219 N17	-	5.0
DAE219 N18	-	0.625
DAE219 N19	-	1.25
DAE219 N20	N/A*	1.25
WT control		0.3125
ompC KO		0.625

Sequencing of *ompC* genes from cefotaxime and T4 phage resistant colonies. 1 mL of log-phase cultures of multiple cefotaxime and T4 phage resistant colonies grown overnight in LB were suspended in 1.5 mL tubes, centrifuged at 10,000 RPM for 1 min and the supernatants were discarded. The pelted cells were then resuspended with 1 mL dH_2O and incubated in 95 ºC water bath for 10 mins. The heated cultures were then centrifuged at 8000 RPM for 10 mins and the supernatant (containing the genomic DNA) was transferred to new tubes. WT BW25113 was used as a PCR positive control. The PCR reaction was set up by the method of Chang et al. to amplify the *ompC* gene with the Bio-Rad T100 Thermal Cycler using Platinum Taq DNA polymerase and buffer (Invitrogen) (1). See Table 2 and 3 for the PCR primers and conditions. The PCR products and a 1kb DNA ladder (Invitrogen) were electrophoresed on a 1% (w/v) agarose gel containing SYBR Safe DNA Stain in 0.5X TBE (Invitrogen) at 110V in 1X TBE. The gel was then visualized under UV light with the Alpha Innotech MultiImage light cabinet. After confirming the presence of amplicon bands, PCR products were then cleaned by GeneJET PCR purification spin column kit (ThermoFisher). The cleaned products concentrations were determined by Nanodrop and diluted to 4 ng/µL using sterile dH2O. 10 µL of this diluted PCR product along with 5 µM forward and reverse (separate primer tubes) were sent to GENEWIZ sequencing center to Sanger sequence the full gene of *ompC*.

Analyzing *ompC* sequences. The sequences were checked for quality and complete resolved sequences were constructed using the overlap of the forward and the reverse sequences. The resolved sequences were then aligned using MUSCLE (Multiple Sequence Comparison by Log-Expectation) algorithm tool. Wild-type *ompC* obtained from *E. coli* BW25113 genome (deposited in Genbank database) was used as a reference. Aligned sequences were compared with the reference for mutations using the Jalview software. Manual curation was performed to ensure correct alignment. See supplemental for *ompC* sequences. Amino acid sequences of those mutant sequences were obtained using ExPASy translation tool and confirmed with blastx (Nucleotides to Proteins Basic Local Alignment Search Tool) and mapped to *ompC* gene of *E. coli* BW25113. Predicted 3D structure for the mutant sequence was constructed using SWISS-MODEL modeling server and a PDB file was obtained and visualized using PyMOL software. A superimposed model of our mutant OmpC onto wild-type OmpC was created using the Vector Alignment Search Tool (VAST).

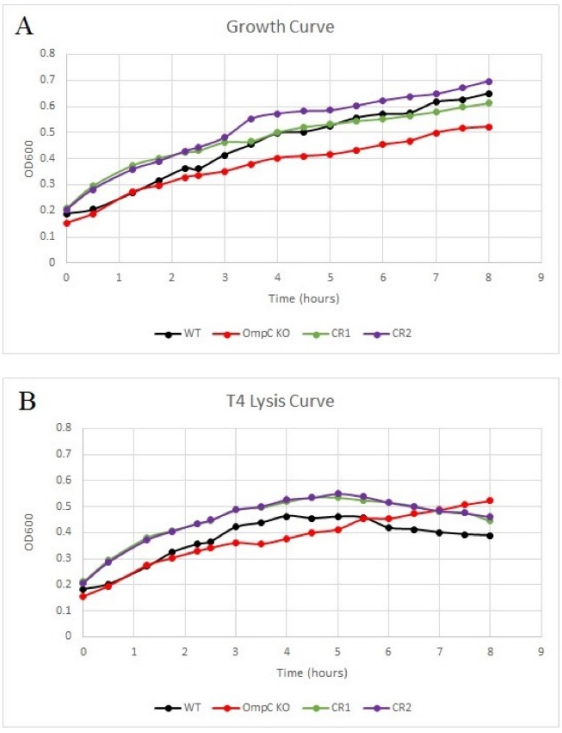


FIG. 1 Growth/lysis curve of BW25113 (WT) , JW2203-1 (OmpC KO) and CTX resistant (CR) cultures 1 and 2. Data shown represents OD600 readings of *E. coli* suspended in 200 μL LB. Readings were taken on BioTek Epoch Microplate Spectrophotometer every 30 min for 8 hours total. (A) Growth curve control of each strain (n=1). (B) Lysis curve of each strain (n=3), T4 phage added at 3 hours at MOI = 0.1.

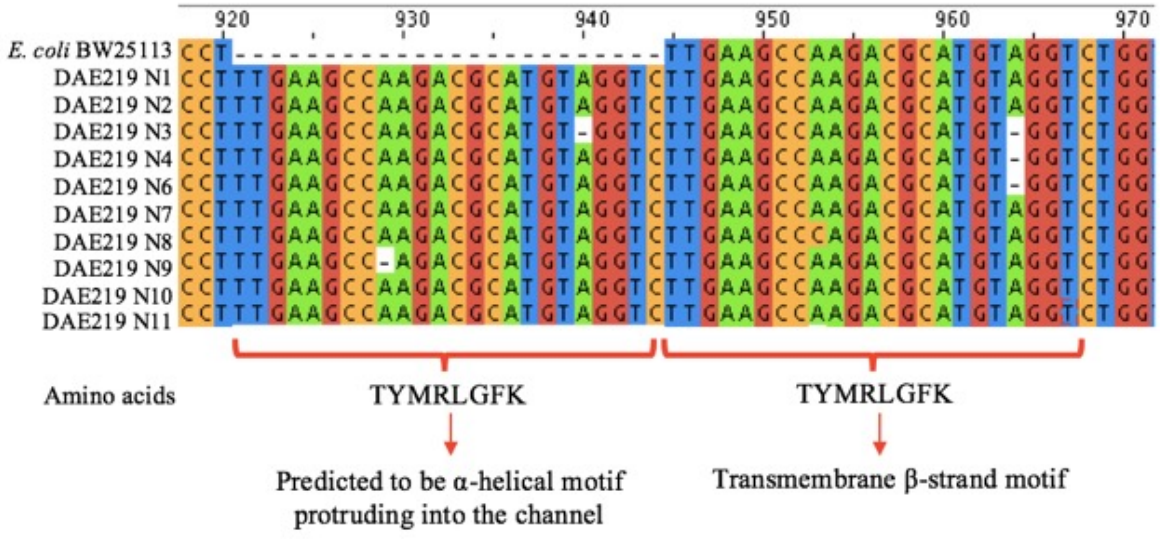
RESULTS

ompC* knockout (JW2203-1) shows higher MIC to cefotaxime compared to wild type (BW25113) *E. coli. As previous research has not addressed the implications of cefotaxime resistance in an OmpC knock out model, we tested whether the lack of this protein channel would show a phenotypic difference between wild type (BW25113) and *ompC* knockout (JW2203-1). We conducted an MIC test using a cefotaxime (CTX) gradient suspended in high sucrose (20%) M9 media (M9S). The gradient was achieved by serially diluting cefotaxime by a factor of 2 to yield the following concentrations: 0.15625, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20 µg/mL CTX. In each plate we also included a no antibiotic positive control and a no inoculum negative control. From two replicates of each *E. coli* strain, we found that the *ompC* knockout strain was able survive in 2.5 µg/mL CTX whereas the wild type strain did not survive past 0.3125 µg/mL CTX. These results were then replicated in later MIC tests, demonstrating that without OmpC, *E. coli* can survive up to 6-folds higher exposure to cefotaxime antibiotic.

Sequential passaging of *E. coli* BW25113 in increasing cefotaxime selects for genomic antibiotic resistance. To test whether cefotaxime exposure can select for mutations in the *ompC* gene, we seeded *E. coli* BW25113 in to 96 well plates containing 200 µL of M9S with increasing concentrations of cefotaxime by 0.05 µg/mL. Through sequential passaging of cultures showing sufficient growth (OD600 > 0.1) from the highest concentration wells in to new gradients starting from just below the previous MIC, we selected for two batch cultures that showed resistance comparable to the *ompC* knock out phenotype, at 1.35 µg/mL CTX.

To confirm that this resistance phenotype is due to the genetic selection rather than an adaptive response from prolonged exposure to antibiotic stress, we passaged the two CTX resistant cultures (CR1 and 2) into LB broth without antibiotic and repeated the initial MIC test. Both CR1 and 2 survived at 2.5 µg/mL CTX. Results from our wild type and *ompC* knockout controls were consistent with that of the initial MIC: *ompC* knockout (JW2203-1) grew to 2.5 µg/mL CTX and wild type (BW25113) grew to 0.3125 µg/mL CTX. These results confirm that the resistance seen in our selected cultures is retained, suggesting that resistance was produced by a genetic difference rather than adaptive response.

FIG. 2 Sequence alignment of mutant against wild type BW25113 *ompC* gene. Insertion mutation was observed in 7 unique isolates - DAE219 N1-4 and DAE219 N6-11 (DAE219 N1-4 are replicates of the same isolate). The inserted region is translated to 8 amino acids (3'-5' 2nd ORF) - T63, Y64, M65, R66, L67, G68, F69, K70, and appears to be a duplication of the adjacent region which encodes for a transmembrane β-strand motif.



Cefotaxime resistant isolates display trends of delayed lysis by T4 phage. After confirming genetic cefotaxime resistance, we tested whether our selected cultures would have a difference in T4 phage susceptibility. We performed a growth/lysis curve for 8 hours as shown in Figure 1. Results from both curves show that *ompC* knockout grew at a slower rate as compared to other strains. Additionally, both selected CTX resistant cultures (CR 1 and 2) showed faster initial growth than wild type and *ompC* knockout strains. For the lysis condition, we allowed bacterial growth to reach an OD600 of approximately 0.4 before adding in T4 phage (MOI = 0.1) at 3 hours. As shown in Figure 1B, when OmpC is absent, no lysis occurs. Both CTX resistant cultures (CR1 and 2) showed delayed initiation of lysis (Time = 5 h) as compared to wild type (Time = 4 h). When comparing against their respective non lysis controls, a possible reduction in lysis, is seen in CR1 as compared to wild type. However, due to the limitations in equipment, we were not able to gather enough data to confirm whether this trend is pervasive throughout the lysis process or restricted to solely the first 5 hours. Nevertheless, T4 treated cultures were left for 48 hours after initial phage introduction and isolates resistant to both cefotaxime and T4 phage were obtained for sequencing.

Cefotaxime and T4 phage double resistant isolates have insertion mutation in *ompC* gene. After T4 phage challenge, double resistant cultures from CR 1 and 2 wells were plated on M9S agar plates to obtain isolated colonies. Based on successful PCR amplification of the *ompC* gene, 20 colonies (DAE219 N1-20) were selected and sent for DNA sequencing along with wild type BW25113 to screen for potential mutations in the *ompC* genes. We included 4 replicates of the sample clone (DAE N1-4) to ensure DNA sequencing quality and consistency. DAE219 N5 and N20 sequencing reactions failed and therefore will not be considered. When aligned against BW25113, we observed an insertion mutation in the *ompC* gene of 10 out of the 18 samples sequenced as shown in Figure 2. The sequence of the insert was consistent between all 10 mutants. The validity of this

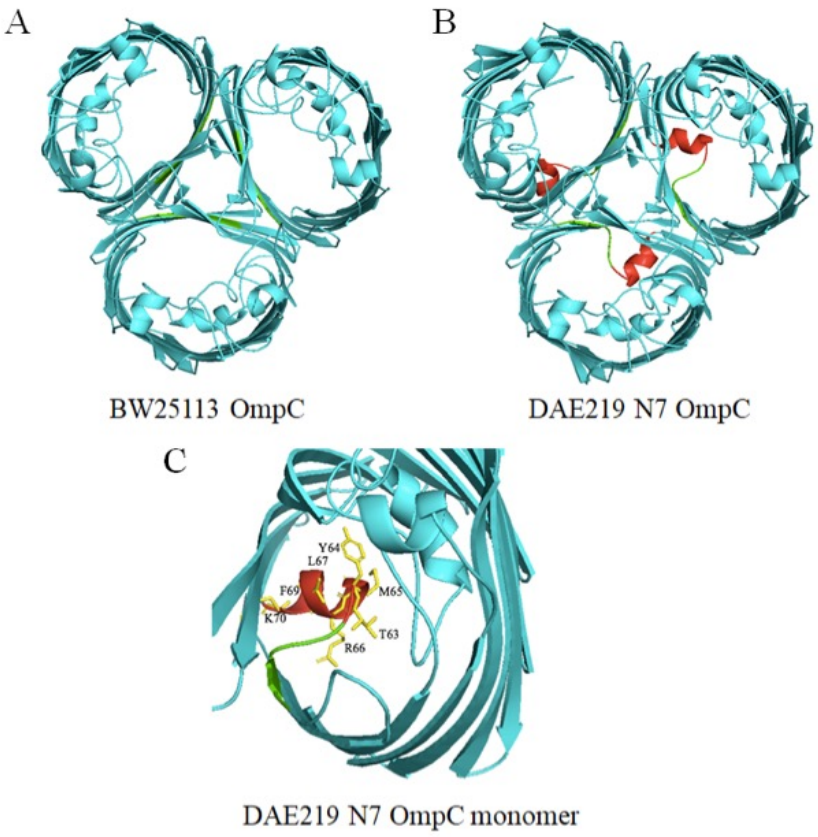


FIG. 3 Three-dimensional model of wild-type and mutant OmpC from extracellular view. Images were generated using PyMOL software (A) 3D structure of wild type OmpC from *E. coli* BW25113. (B) Predicted structure of our identified mutant OmpC using Swiss-Model web-based modelling server. The inserted region in the mutants is coloured red and folds into α-helical motif compared to the same sequence region adjacent to it that folds into β-strand motif (green). (C) Shows the relative spatial view of the amino acid side chains of this inserted region within each pore, relative positions of the 8 inserted amino acid residues are indicated.

insertion was confirmed by consistency between DAE N1-4 sequencing. Additionally, 5 samples (2 with and 3 without mutations) were sequenced forwards and backwards to resolve the full *ompC* sequence of these isolates and validate that insertion sequences overlapped consistently between both sequencing orientations.

As shown in Figure 2, the mutations detected appears to be a duplication of 8 amino acids (63 to 70) of an adjacent sequence downstream found in wild type BW25113, which encodes a transmembrane β-strand motif. Using Swiss-Model web-based modelling server, we constructed a predictive 3D protein structure model of this mutant OmpC. In this model, the duplicated region folds into α-helical motif protruding into the pore channel closer towards the periplasmic side as shown in Figure 3B. Figure 3A shows the wild-type *ompC* which lacks this motif. When we superimpose both models (mutant and wild-type) using VAST, we again observed this motif unique to our mutants as shown in Figure 4.

***E. coli* with mutant OmpC has high resistance to cefotaxime.** To look at individual differences in cefotaxime resistance between our double resistant isolates (DAE219 N1-20) we performed an MIC for each isolate. We kept the same dilution factors but narrowed the MIC range from 0 to 5 µg/mL CTX due to limited resources. As shown in Table 5, 3 out of 8 of the isolates that carried our identified mutation grew to 5 µg/mL CTX. On average, the isolates carrying the insertion mutation had a higher MIC but on an individual level, the presence of mutation was not predictive of the concentration of cefotaxime each isolate could tolerate. This result suggests that the *ompC* mutation identified may contribute to cefotaxime resistance but there are likely other contributing factors as well. Another factor to note is that in this MIC, we observed ompC knockout JW2203-1 to be susceptible at a much lower concentration than previously observed, and all data shown represents n=1, therefore the data shown should be interpreted with caution.

DISCUSSION

With the increased prevalence of multi-drug resistant bacteria posing threat, clinical researchers are turning to phage therapy as an antibiotic alternative. In this study we've provided evidence for a potential link between antibiotic and phage resistance in an *E. coli* model. Since antibiotics and phages may share target structures in bacteria, mishandling of antibiotics may have more disastrous results than previously anticipated, and phage therapy may be limited in efficacy.

In this study, we used an *E. coli* model to address the understudied relationship between antibiotic and phage resistance. We first established that lack of OmpC porin protein yields resistant phenotypes for both cefotaxime antibiotic and T4 phage. Although previous literature has identified the relevance of mutant OmpC in cefotaxime resistance, to our knowledge, no study has published the effect of *ompC* knockout in *E. coli* (4, 9) . Our

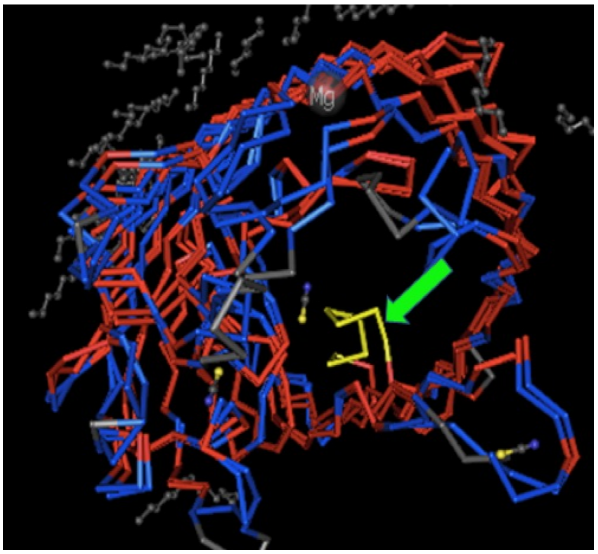


FIG. 4 Superimposed model of wild type and predicted mutant OmpC structure. Image created using the Victor Alignment Search Tool (VAST). Our identified insertion, coloured in yellow, could not be matched to any region in the wild-type structure.

results showing a 6-fold increase in cefotaxime resistance in *ompC* knockout compared to wild type *E. coli,* therefore, contributes a novel finding to the literature and can be used as a baseline comparison.

Previous research by Lou et al. have denoted the relevance of OmpC mutations in cefotaxime resistance (9). Extending from this find, we aimed to see whether cefotaxime exposure could select for mutations in *ompC* that also conferred resistance to T4 phage. The results we've presented supports this hypothesis, as exposing *E. coli* BW25113 to both antibiotic and phage challenge selected for an insertion mutation that was consistently observed in several of our mutant *ompC* sequences. This mutation, however, was different from that reported by Lou et al. The clinically reported cefotaxime resistant mutants (OmpC20 and OmpC33) had missense mutations, replacing 3 amino acid residues in the structure (9). Through our selection process, we found a duplication of 8 amino acids, normally found in an adjacent region encoding a conserved β-strand motif of OmpC. Using Swiss-Model web-based modelling server, the duplicated region was predicted to fold into α-helical motif instead, protruding into the core of each of the three channels. A similar mutation was found in cephalosporin resistant *Enterobacter aerogenes* by Thiolas et al. (4). In their L5 mutants, a 11-12 residue insertion was identified in the extracellular loop region of OmpK26, a homologue of OmpC, however, the authors did not discuss how this mutation may have conferred resistance (4).

Currently, the effect of our identified insertion on OmpC function has not been explored but potential explanations include: 1) disruption of tertiary or quaternary structure, sterically preventing molecule transport and phage attachment 2) disruption of charge and hydrophobicity of the porin, limiting ability of porin to interact with molecules such as antibiotics or DNA to facilitate transport. Of the 8 duplicated amino acid residues, 2 are positively charged: R66 and L67, which may create salt bridges with the negatively charged residues lining the other side of the barrel wall, and consequently constrict the pore. Other amino acids such as Y64 and F69 create less space in the pore due to their bulky aromatic ring side chains. Additionally, F69, M65, and L67 are hydrophobic and may perturb the electrostatic field of the pore.

Conclusions The results of this study prompt further investigation of the role of OmpC in the relationship between cefotaxime and T4 phage resistance. Currently, our data suggests that a relationship may exist but there are a number of limiting factors that need to be addressed to form a causal conclusion.

Firstly, our study only partially captured the effects of the mutant OmpC on T4 lysis. The lysis curves shown in Figure 1B represents batch cultures taken from cefotaxime resistant wells. Although a trend suggestive of resistance is seen in CR1 in the first 5 hours after phage introduction, it is unknown whether this difference is significant, whether it persists throughout the lysis period, and whether this was a result of reduction of lysis or a delay in lysis. Additionally, once double resistance isolates were identified, an MIC was performed on individual isolates DAE219 N1-20 but we were unable to perform a lysis analysis to confirm differences of phage susceptibility between those containing the insertion mutation and those that did not. Although these selection measures favoured the identified mutation, we cannot claim that this mutation confers resistance to T4 phage.

Secondly, our conclusions were made from a small sample size. We cannot be certain that the selection process consistently favours the selected mutation until more biological replicates are performed. Sequencing was only performed on 20 colonies, 4 of which from the same isolate. This captures a small subset of double resistant bacteria produced from our selection process to provide proof of principle. A wider screen of sequences is needed to accurately determine the frequency and variety of *ompC* mutations.

Lastly, although a relationship may exist, a complementation study would be necessary to see if the phenotypes are indeed connected through OmpC, namely does resistance to both agents decrease when wild type OmpC is reintroduced into our isolated mutants? As seen from Table 5, there could be mutations in other genes we have not yet identified contributing to the resistance phenotype of our selected mutants. Therefore, it is important

to test whether concordant resistance to cefotaxime and T4 phage is truly related through OmpC or if it this is due to simultaneous mutations in other structures.

Future Directions As previously discussed, limitations to this study includes a need for more T4 phage lysis data and biological replicates. As such, our future aims include amending our current T4 lysis data and repeating the experiment thus far to see whether consistent mutation to *ompC* could be produced through our selection process. The T4 phage lysis curve we've presented was from two batch culture of cefotaxime resistant isolates, however this lysis pattern is not representative of the identified mutants. Therefore, the next step will be generating individual lysis curves for DAE219 N1-20 and to examine whether 1) These "double resistant" isolates have a different lysis pattern than wild type BW25113 and 2) Whether the isolates containing insertions in *ompC* show higher resistance to T4 lysis than those that do not.

In the study by Lou et al., complementation of wild type *ompC* in to cefotaxime resistant strains lowered the MIC (9) . To confirm OmpC involvement in the relationship between cefotaxime and T4 phage, we plan to perform a similar complementation assay. The assay will consist of cloning wild type *ompC* into a plasmid vector and transforming this plasmid into our mutants. If the insertion mutation we've identified in the *ompC* gene of our double resistant isolates is indeed responsible for our observed resistance phenotype, complementation of wild type OmpC would lower both the MIC of cefotaxime and accelerate T4 phage lysis.

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