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Exploring the role of OmpC deletion and O-antigen expression in T4 bacteriophage-induced lysis of *Escherichia coli* K12 cells.

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SUMMARY Escherichia virus T4 (T4 phage) is a species of bacteriophage that infects *Escherichia coli* (*E. coli*) bacteria. Resistance to T4 phage infection has been found in *E. coli* strains that express the outermost component of lipopolysaccharide, O-antigen, or in those that do not exhibit outer membrane porin C (OmpC). However, the exact mechanism by which resistance is conferred in both cases has yet to be elucidated. *E. coli* K12 strains lack O-antigen and are susceptible to T4 phage infection due to a mutation in *wbbL,* a rhamnosyltransferase gene necessary for O-antigen synthesis. As such, we sought to explore the combined effect of *ompC* deletion and O-antigen expression in T4 bacteriophage-induced lysis by creating a plasmid that contains the *wbbL* gene, followed by its introduction into an *ompC* knock-out strain. Using molecular cloning techniques, an intact copy of *wbbL* was inserted into a plasmid vector as confirmed by restriction enzyme digestion, gel electrophoresis, and Sanger sequencing. The plasmid, designated pCR2.1-*wbbL-α,* was then transformed into wild-type (BW25113) and *ompC* knock-out (JW2203-1) strains and evaluated for resistance against T4 phage using a qualitative assay that detects bacteriophage-induced zones of clearance. Transformation of both strains with pCR2.1-*wbbL-α*, demonstrated no zones of clearance, suggesting cells were rendered resistant to T4 infection. Contrary to the literature, o*mpC* knock-out cells demonstrated zones of clearance similar to the WT strain, suggesting that deletion of *ompC* on its own is not sufficient to confer resistance to T4 phage infection. pCR2.1-*wbbL-α* can be used in future studies by introducing O-antigen into other *wbbL* deficient strains to further explore the role of O-antigen mediated bacteriophage resistance.

INTRODUCTION

Escherichia coli K-12 has become the go-to experimental model in molecular biology, enduring constant passaging and exposure to mutagens, leading to an accumulation of various mutations and differentiating its genome from other wild-type strains (1). One such mutation is the transposable insertion 5 (IS5) mutation in the *rfb* locus which disrupts the *wbbL* gene (2). *wbbL* codes for the rhamnosyltransferase, responsible for creating sugar linkages in the oligosaccharide chains (O-antigen) in lipopolysaccharide (LPS) (3). Thus, *E. coli* K-12 has lost its ability to synthesize O-antigen and presents incomplete LPS on its cell surface.

Bacteriophage range is determined by the presence of specific host cell receptors displayed on the cell surface, which are hijacked by the phage to trigger phage adsorption into the cell (3). For instance, it has been shown that O-antigen deficient strains such as *E. coli* K-12, are susceptible to infection by T-even bacteriophages such as T4 and T7 (4–6). Conversely, reconstitution of *wbbL* and the subsequent restoration of O-antigen synthesis in *E. coli* K-12 derived strains results in decreased T4 and T7 bacteriophage mediated lysis. This suggests that the presence of O-antigen can provide a protective mechanism against phage infection (2, 7, 8).

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Outer membrane protein C (OmpC) is a transmembrane pore-forming protein located in the outer membrane of the *E. coli* cell and is involved in the passive transport of small molecules such as nutrients, salts and antibiotics molecules (9,10). OmpC has been described to be an important cell receptor for phage adsorption, and its removal has been shown to render *E. coli* more resistant to infection (9, 11).

Morgan *et al*. explored the effects of O-antigen synthesis and *ompC* deletion (Δ*ompC*) on T4 viral adsorption and phage-induced lysis of *E. coli* K-12 derived cells (12). Using transmission electron microscopy, they found that there was considerably less membrane-bound T4 phage on the surface of DFB1655 L9 (a *wbbL*$^+$ K12 strain), and JW2203-1 (an *ΔompC* K12 strain), when compared to the wildtype strain, MG1655. Using a lytic assay, the authors demonstrated that both DFB1655 L9 and JW2203-1 were initially resistant to T4 phage-induced lysis. However, upon long-term incubation with the phage, a substantial drop in optical density ($O.D_{600}$) was observed, suggesting that both strains appeared to lose resistance. This suggests that the independent presence of O-antigen or the absence of OmpC in *E. coli* K-12 cells may only provide partial resistance to T4 phage infection. O-antigen may provide an incomplete barrier and still allow viral interactions via OmpC, while Δ*ompC* may still allow viral interactions via other cell-surface receptors such as LPS. This is supported by Washizaki *et al*. who observed that in the presence of OmpC, T4 phage adsorption was successful with non-specific interaction with LPS, while in the absence of OmpC, T4 phage adsorption required interactions with specific glucose residues in LPS (13). These observations led Washizaki *et al*. to propose two models of infection: the OmpC dependent and independent pathway. These findings have only been observed in K-12 strains which do not express O-antigen, suggesting its expression may be sufficient in preventing specific interactions between the phage and the LPS outer-core sugars in the OmpC independent pathway. Therefore, restoration of O-antigen synthesis in *ΔompC E. coli* cells will likely inhibit both the OmpC dependent and independent pathways of phage adsorption, thus resulting in increased resistance to infection by T4.

This paper evaluated the combined effect of OmpC knock-out and O-antigen expression in T4 bacteriophage resistance of *E. coli* K-12 derived cells. Using molecular cloning techniques, pCR2.1-*wbbL-α*, a plasmid containing an intact copy of *wbbL* was transformed in an *ΔompC E. coli* strain (Fig 1), producing an *ΔompC wbbL+* phenotype which was assessed for resistance to T4 bacteriophage resistance. We found that the combination of *ompC* deletion and O-antigen restoration prevented phage from inducing the bacteriolysis of *E. coli* cells. However, bacteriophage resistance appeared to be reliant on O-antigen restoration alone and independent of *ompC* deletion. More sensitive, quantitative studies will be needed to confirm these findings. Using pCR2.1-*wbbL-α,* future studies will now be able to introduce O-antigen into other *wbbL* deficient strains to further explore the role of O-antigen mediated bacteriophage resistance.

METHODS AND MATERIALS

Bacterial strains used. *E. coli* K-12 derived strains WG1, BW25113 (Keio Collection parent strain), JW2203-1 (*ompC* knock-out strain from the Keio Collection) and DFB1655 L9 (*wbbL* restored *E. coli* K-12 strain engineered by Browning *et al*. (2)) were obtained from the MICB 401 culture collection of the Microbiology and Immunology Department at the University of British Columbia. One Shot™ TOP10 chemically competent *E. coli* cells were purchased from Thermo Fisher Scientific (cat. K450002). Genotypes for all *E. coli* strains are further described in Supplementary Table 1 (Table S1). After streaking from glycerol stock solutions, bacterial colonies were maintained at 4°C, on 1.5% LB-agar plates with the appropriate antibiotic. For use in experiments, overnight cultures were prepared by inoculating single colonies in 5mL of LB broth with the appropriate antibiotic and incubating at 37°C for 12-16 hours. T4 bacteriophage was provided courtesy of our colleagues in the MICB 401 lab from the University of British Columbia.

Preparation of media and reagents. Stock solutions of 5mg/mL kanamycin sulphate (Gibco), 100mg/mL ampicillin sodium salt (Fisher Scientific), ampicillin anhydrous (Fisher Scientific), 1M $CaCl_2$ (VWR), 200mg/mL X-gal (Invitrogen), Luria Bertani (LB) (tryptone (Fisher Scientific), yeast extract (Fisher Scientific), NaCl (Sigma-Aldrich), distilled water)

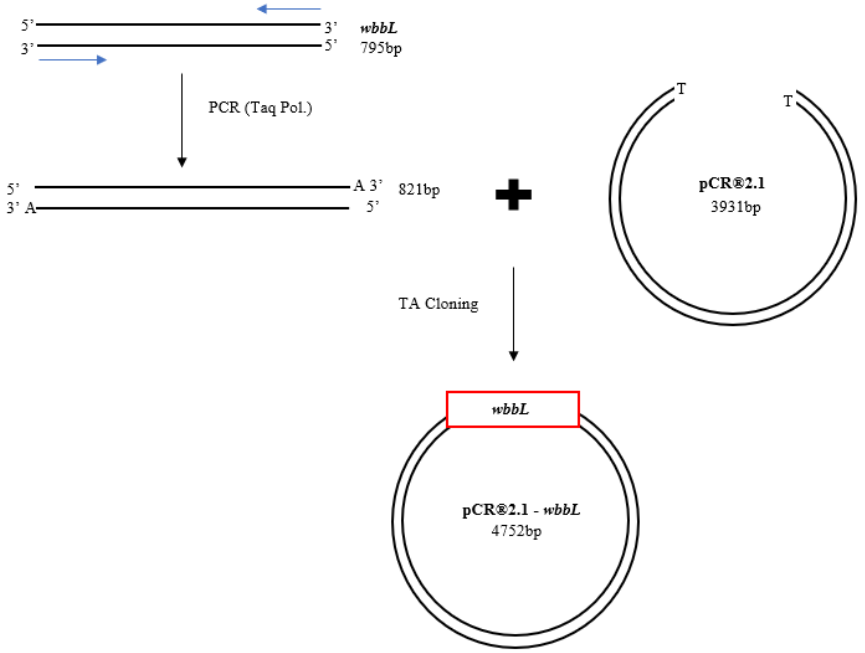

FIG. 1 TA cloning workflow. Depiction of the steps involved in the construction of plasmid pCR2.1-*wbbL*. *wbbL* was amplified using primer sequences obtained from Browning *et al*. (2). Taq polymerase was used to generate 3' adenylated PCR products for subsequent TA cloning. *wbbL* was inserted into linear pCR2.1 plasmid vectors via TA cloning reactions. A more detailed pCR2.1 plasmid vector map with restriction enzyme digest sites can be found on the Addgene vector database (21).

broth and LB agar (Invitrogen) were prepared following standard protocols. For antibiotic selection, a final concentration of 50µg/mL of kanamycin, 100µg/mL of ampicillin sodium salt, or ampicillin anhydrous was added to LB-agar plates. For blue-white screening, in addition to the selection antibiotic, X-gal was added to a final concentration of 20µg/mL.

Touchdown Polymerase Chain Reaction (PCR) amplification of *wbbL* from *E. coli* strain K-12 WG1. Genomic DNA (gDNA) was extracted from an overnight culture of *E. coli* K-12 WG1 using the EZ-10 Spin Column Bacterial Genomic DNA Miniprep Kit by BioBasic (cat. BS423) according to the manufacturer's instructions. PCR amplification of the *wbbL* gene from the gDNA was performed using the Maxima Hot Start Taq DNA Polymerase by ThermoScientific (cat. EP0601) following the manufacturer's instructions. *wbbL*-specific primer sequences were designed by Browning *et al*. (2) and ordered from Integrated DNA Technologies (IDT). A final concentration of 0.4µM of each primer was used per reaction sample. Primer sequences are outlined in Supplementary Table 2 (Table S2). PCR amplification of the beta-lactamase gene from the pUC19 plasmid was used as a positive control. Touchdown PCR was performed using the Bio-Rad T100™ Thermal Cycler with the following parameters: 4 min of denaturation at 95°C, followed by 6 cycles of 30 sec of denaturation at 95°C., 45-sec annealing at 61°C decreasing 1°C per cycle to 53°C on the last cycle, then 2.5 min extension at 75°C. This was followed by 28 cycles of 30 sec at 95°C, 45 sec at 53°C, and 2.5 min at 75°C. A final extension for 10 min at 75°C was included to ensure that all PCR products were full length and 3′ adenylated. For downstream application, PCR products were then purified using the PureLink PCR purification Kit by Invitrogen (cat. K3100-01) following the manufacturer's instructions. To determine DNA concentrations, UV absorbance was measured using the NanoDrop™ Spectrophotometer.

Agarose Gel Electrophoresis. Samples were diluted in 6X Orange DNA Loading Dye (ThermoScientific, cat. R0631) and ran on a 1% agarose gel with RedSafe™ Nucleic Acid Stain (LiliF Diagnostic, cat. 21141) in 1X TAE buffer at 100V for 50min. O'GeneRuler DNA Ladder Mix by ThermoScientific (cat. SM1173) was used as a size reference. The gels were visualized using the BioRad ChemiDoc imaging system.

Construction of pCR2.1-*wbbL* plasmid. Using the TOPO™ TA™ cloning kit by Invitrogen (cat. K450002), the purified *wbbL* PCR product was cloned into the linearised pCR2.1-TOPO vector following the manufacturer's instructions. A vector-to-insert molar ratio of approximately 7:1 was used for the cloning reaction. The reactions were incubated at room

temperature for 5 min. and immediately used for subsequent transformation into OneShot™TOP10 Chemically Competent *E. coli* cells.

Transformation and screening of chemically competent *E. coli* with pCR2.1-*wbbL*. Chemically competent *E. coli* were incubated on ice for 5 min with either pCR2.1-*wbbL* from the cloning reaction or the appropriate controls. The cells were then heat-shocked for 30 sec at 42°C. To allow for recovery, Super Optimal broth with Catabolite repression (S.O.C) media or LB broth was added to the samples and incubated at 37°C with shaking at 200 RPM for 1 hour. Undiluted, 1:10, 1:100, and 1:1000 dilutions were spread-plated onto LB-Agar plates with 100µg/mL ampicillin and 20µg/mL X-gal. White colonies were selected and streaked onto fresh LB-agar ampicillin plates.

Orientation and sequence analysis of pCR2.1-*wbbL* insert. Isolation of pCR2.1-*wbbL* from an overnight culture of transformed OneShot™TOP10 cells was performed using the EZ-10 Spin Column Plasmid DNA MiniPrep Kit by BioBasic (cat. BS614) following the manufacturer's instructions. To determine the insert orientation, a single enzyme restriction digest was performed using HindIII (New England BioLabs, cat. R0104) according to the manufacturer's instructions. Following digestion, the enzyme was heat-inactivated by incubating at 80°C for 20 min and stored at 4°C until the next day. The digested plasmid and undigested controls were then run on a 1% agarose gel and subsequently visualized as previously described. To verify the sequence of the *wbbL* insert, each isolated plasmid sample was sent to GeneWiz for Sanger sequencing using the T7 promoter, with M13 forward and reverse primers provided by the sequencing facility. Sequencing results were aligned in SnapGene (version 6.0.2) to the *Escherichia coli* K12 WG1 *wbbL* sequence (accession number: AAB49382.1) from the European Nucleotide Archive (ENA) database.

Bacterial strain construction. To prepare chemically competent BW25113 and JW2203-1 (Δ*ompC*) cells, overnight cultures were subcultured and grown to an $O.D_{600}$ of around 0.4 and chilled on ice for 20 min., then pelleted and washed with ice-cold 0.1M $CaCl_2$. To construct various bacterial strains, the chemically competent cells were heat-shock transformed as described above with pCR2.1-*wbbL-α*, pCR2.1-*wbbL-β*, or the plasmid vector. 1:10 and 1:100 dilutions of the cells were spread-plated onto LB-agar plates with 100 µg/mL ampicillin and incubated overnight at 37°C. Vector diagrams were generated using SnapGene (version. 6.0.2).

Stab Assay. DFB1655 L9 and transformed BW25113 and JW2203-1 cells were incubated at 37°C in LB-broth with the appropriate antibiotic while shaking at 200 RPM for 4 hours. The liquid culture was then spread-plated onto LB-agar plates with the appropriate antibiotic. The back of a sterile swab was dipped into T4 phage stock, then stabbed into the LB-agar plate consecutively, beginning at the 12 o'clock position, and stabbed clockwise for a total of 6 times, equally spaced apart (n = 1).

RESULTS

An 800 base pair PCR product was amplified from *E. coli* K-12 WG1 genomic DNA. PCR amplification was performed using isolated genomic DNA from *E. coli* K-12 WG1 as the template to obtain an undisrupted *wbbL* gene. Unlike most laboratory strains, the *wbbL* gene in *E. coli* K-12 WG1 does not contain an IS5 insertion. Instead, O-antigen synthesis is disrupted via a deletion in the upstream end of the O-antigen (*rfb*) gene cluster (14). Before PCR amplification, the primer sequences sourced from Browning *et al*. were analyzed using the Multiple Primer Analyzer web tool by ThermoFisher which revealed a 10°C melting temperature difference between forward and reverse primers (2, 15). Incompatible annealing temperatures (ΔTm <5°C) may lead to non-specific annealing, so touch-down PCR was performed to increase primer binding specificity (16). To further optimize the PCR reaction, three different volumes of template DNA were used: 2µl (120ng), 1µl (60 ng) and 0.5µl (30 ng). The size and purity of the resulting PCR products were assessed with 1% agarose gel electrophoresis and revealed faint bands at approximately 800 base pairs (bp) for all sample conditions (Fig 2). The negative control, which corresponded to a PCR reaction without

template DNA, yielded no bands on the gel. Of the two positive controls analyzed, the first yielded a ~200 bp band and the second, a ~750 bp band. Taken together, this indicates that *wbbL* was indeed PCR-amplified from the *E. coli* K-12 WG1 genome. Of the three PCR samples, NanoDrop readings for Sample 3 revealed the highest purity, with a 260/280 ratio of 1.9 and a sharp peak at 260 nm on the UV-Vis absorbance spectra, and chosen for downstream plasmid construction.

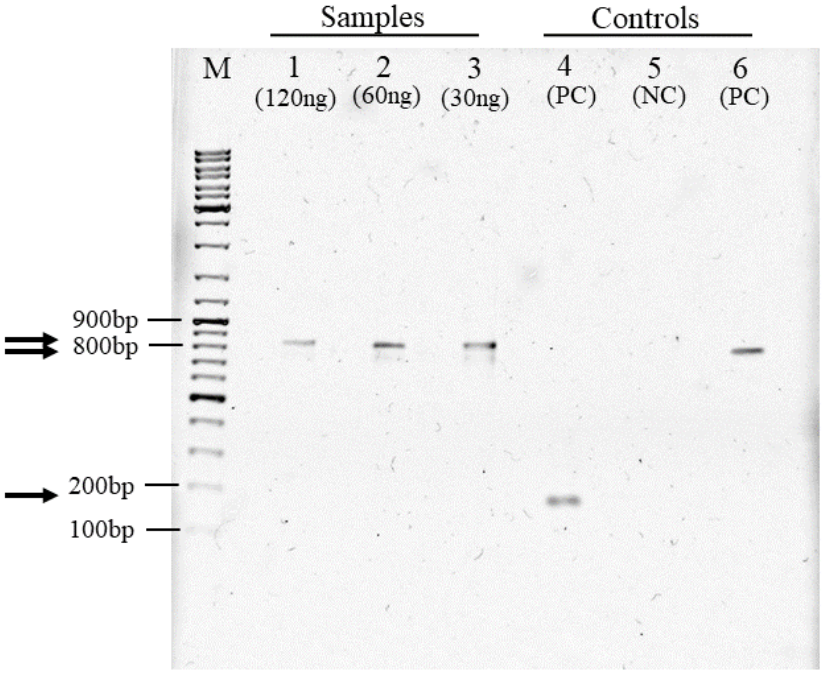

FIG. 2 PCR amplification of the *wbbL* gene from *E. coli* K-12 WG1 gDNA yields an ~800 bp product. 1% agarose gel electrophoresis of PCR amplified products. M refers to the molecular marker. Sample lanes 1, 2, and 3 correspond to amplification reactions using 120 ng, 60 ng, and 30 ng of the template DNA respectively. Sample lanes 4-6 correspond to positive (PC) and negative (NC) controls. Expected band sizes for samples 1, 2, and 3 (upper arrow), positive control (middle arrow), and negative control (lower arrow) are shown for reference.

Transformation with pCR2.1-*wbbL* yields two colonies. To determine the success of plasmid construction, OneShot™ TOP10 *E. coli* cells were transformed with pCR2.1-*wbbL* cloning products. Transformants were selected using ampicillin and blue-white colony screening. Ampicillin prevented the growth of untransformed *E. coli* cells, while blue-white screening allowed for the identification of colonies that acquired plasmids with an insert. The negative control condition transformed with water showed no growth. The positive control, which was transformed with a plasmid constructed using the TA TOPO kit control DNA, yielded one white colony. The diluted sample conditions (1:10, 1:100, 1:1000) transformed with pCR2.1-*wbbL* saw no growth, whereas two white colonies were observed on the undiluted plate. The two colonies, designated CFU A and CFU B, were restreaked onto fresh ampicillin plates, and subsequently cultured for plasmid isolation.

The *wbbL* gene was inserted in pCR2.1-*wbbL-α* and pCR2.1-*wbbL-β* in opposing directions. TA cloning is a non-directional process and as such, the 3' adenylated PCR product can be inserted into the linear pCR2.1 plasmid vector either in the forward or reverse directions. Thus, HindIII restriction enzyme digestion was performed on plasmids isolated from CFU A (pCR2.1-*wbbL-α*) and CFU B (pCR2.1-*wbbL-β*) to confirm both the presence and directionality of the gene insert. For each colony, a digested and an undigested control condition were performed, and products were subsequently analyzed via 1% agarose gel electrophoresis. This revealed two bands at 4000 bp and 900 bp for CFU A and a single band at 4800 bp for CFU B (Fig 3A). The TA plasmid control, provided with the kit, yielded two products at 4200 bp and 700 bp. The bands observed from CFU A and B suggest the presence of the insert in both colonies and directionality in the forward (Fig 3B), and the reverse orientation, respectively (Fig 3C).

Sequencing results reveal the presence of *wbbL* within the pCR2.1-*wbbL* plasmid. pCR2.1-*wbbL-α* and pCR2.1-*wbbL-β* were Sanger sequenced by GeneWiz to verify the sequence of the insert. Both samples returned valid results with good base quality scores (QS

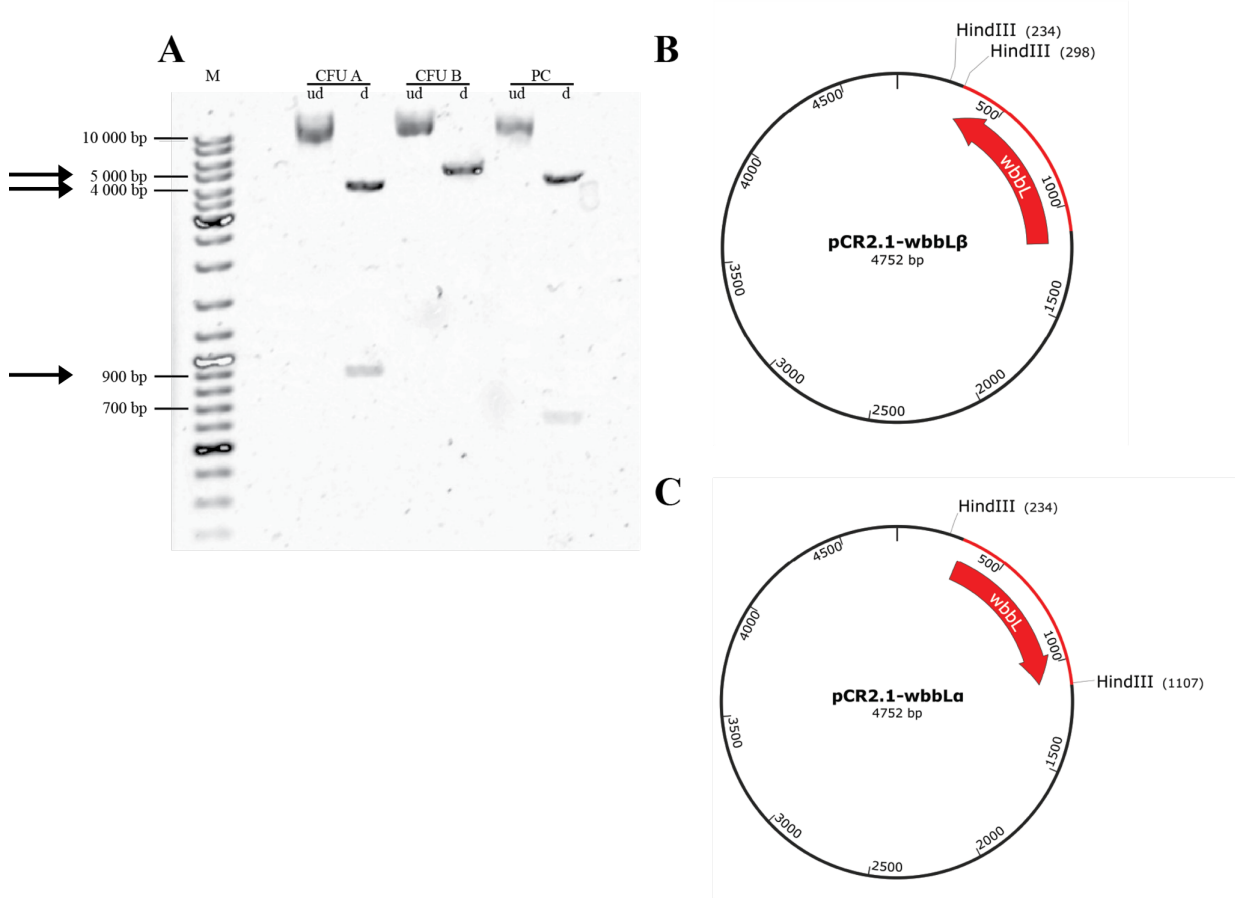

FIG. 3 HindIII restriction enzyme digest on plasmids pCR2.1-*wbbL*-α and pCR2.1-*wbbL-β* reveal the presence of *wbbL* inserts in opposing directions. 1% agarose gel electrophoresis of HindIII restriction enzyme digestion products for plasmids isolated from CFU A, CFU B, and the positive control (PC) (A). M refers to the molecular marker. For each sample, both an undigested control (ud) and digested (d) condition were performed. Expected products for CFU A (arrows 2, and 3) and CFU B (arrow 1) are shown for reference. Vector diagrams outlining the forward (B) and reverse (C) orientation of the *wbbL* insert, along with the location of the HindIII digestion sites have been included for reference.

> 40). Analysis of the forward and reverse reads for both plasmids revealed the incorporation of the insert in opposing directions, consistent with the restriction enzyme digestion results (Fig 3). Alignment of the sequences to the *wbbL* sequence from *E. coli* K-12 WG1 reported in the ENA database (accession no. AAB49382.1) revealed an exact match for pCR2.1-*wbbL*-α (Fig 4, Fig S4, and Fig S5), and two nucleotide mismatches for pCR2.1-*wbbL-β*. According to the SnapGene translation of pCR2.1-*wbbL-β*, the mutations in the insert sequence resulted in an amino acid change from asparagine to aspartate in position 149 (N149D) and an amino acid change from arginine to glutamine in position 226 (R226Q).

Susceptible cells transformed with pCR2.1-*wbbL-α* exhibit resistance to T4 bacteriophage, but remain susceptible when transformed with pCR2.1-*wbbL-β*. pCR2.1-*wbbL-α* functionality was assessed through stab assays on susceptible cells transformed with the plasmid. Stab assays performed on the positive control strain, DFB1655 L9, containing a functional genomic *wbbL*, revealed no zones of clearance. This indicates the functionality of the T4 phage. Assays performed on BW25113 cells yielded zones of clearance, whereas BW25113 cells transformed with pCR2.1-*wbbL-α* yielded no zones of clearance (Table 1, Fig S3). In contrast, assays performed on cells transformed with pCR2.1-*wbbL-β* resulted in observable zones of clearance, demonstrating susceptibility to T4 bacteriophage-induced lysis. The functional pCR2.1-*wbbL-α* was used for downstream experiments.

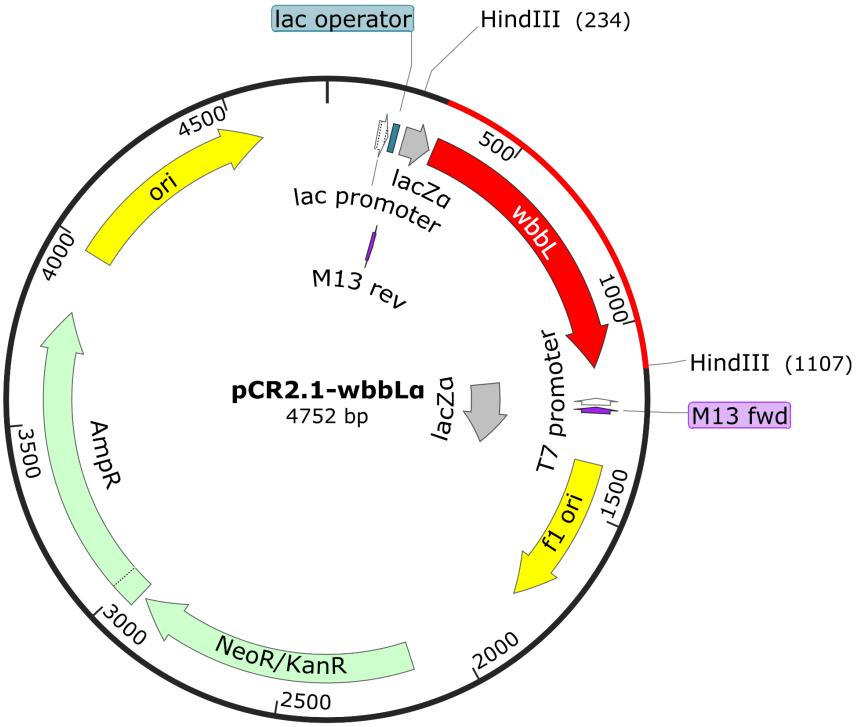

FIG. 4 pCR2.1-*wbbL*-α vector map. The inserted sequence is indicated in red, and the corresponding open reading frame is denoted by the red arrow. Origins of replication for bacteria (ori) and phage (f1 ori) are indicated in yellow. Antibiotic resistance genes AmpR, NeoR, and KanR are indicated in green. The insert-disrupted lacZα gene is indicated in gray. Promoters are represented as white arrows. The M13 forward and reverse sequencing primer sites are indicated in purple. HindIII restriction digestion sites are labeled on the vector. Other restriction sites can be found on the pCR2.1 vector map in the Addgene vector database (21).

No conclusive results for OmpC and O-antigen interactions in T4 bacteriophage resistance following stab assays. The effects of *ompC* deletion and O-antigen expression independently and in combination on T4 bacteriophage resistance were assessed with stab assays performed on three different *E. coli* K-12 derived phenotypes (n = 1): *ompC wbbL*$^+$, Δ*ompC wbbL*$^-$, Δ*ompC wbbL*$^+$(Table 1, Fig S3). The *wbbL*$^+$phenotypes were constructed by transforming either BW25113 or JW2203-1 cells (Δ*ompC*), with pCR2.1-*wbbL-α*. Assays performed on cells transformed with pCR2.1-*wbbL-α* (*ompC wbbL*$^+$ and Δ*ompC wbbL*$^+$ phenotypes) resulted in no zones of clearance. This suggests that *wbbL* expression and subsequent O-antigen synthesis confers T4 bacteriophage resistance in *E. coli* K-12 derived cells. Interestingly, in the Δ*ompC* strain JW2203-1 (Δ*ompC wbbL*$^-$), zones of clearance were observed, suggesting that Δ*ompC* does not significantly influence the resistance of *E. coli* cells to T4 phage.

Table 1. Stab assay results. BW25113 and JW2203-1 strains were transformed with an empty vector or our plasmid constructs, *wbbL*-pCR2.1-*α* and *wbbL*-pCR2.1-*b*, and T4 infection susceptibility was tested through a stab assay. [a] *E. coli* strain. [b] Plasmid used for bacterial transformation. [c] Expected phenotype of the plasmid transformed strains. [d] Results of the stab assay. "Yes" indicates that clearance was observed whereas "No" indicates that no clearance was observed. Images of stab assay plates are located in **Supplemental Figure 3 (S3).**

Strain[a]	Transformed with[b]	Expected Phenotype[c]	Resistance to T4[d] (Yes/No)
BW25113	pCR2.1	*ompC, wbbL*$^-$	No
	pCR2.1-*wbbL-α*	*ompC, wbbL*$^+$	Yes
	pCR2.1-*wbbL-b*	*ompC, wbbL*$^+$	No
JW2203-1	pCR2.1	Δ*ompC, wbbL*$^-$	No
	pCR2.1-*wbbL-α*	Δ*ompC, wbbL*$^+$	Yes
	pCR2.1-*wbbL-b*	Δ*ompC, wbbL*$^+$	No
DFB1655 L9	pCR2.1-*wbbL-α*	*ompC, wbbL*$^+$	Yes

DISCUSSION

In this study, we sought to explore the combined role of O-antigen and Δ*ompC* in T4 bacteriophage-induced lysis of K12 *E. coli.* To do this, we first set out to construct a plasmid

vector containing an uninterrupted *wbbL* gene. Genomic DNA was isolated from *E. coli* K-12 WG1, followed by PCR amplification of the 821 bp *wbbL* gene using primer sequences designed by Browning *et al*. (2). The pCR2.1-*wbbL* vector was subsequently constructed using TOPO TA cloning and transformed into OneShot™ TOP10 *E. coli* cells. This yielded only two colonies which was far lower than expected. Similarly, our positive control yielded only one colony. Low transformation efficiency in both conditions led us to believe that there may have been an issue with the reagents, error in ligation or transformation, or problems within the plates. Analysis of the ligation reaction through PCR amplification followed by gel electrophoresis prior to transformation would help determine whether ligation or transformation was responsible for the low number of observed CFUs. Another possible explanation may be the type of antibiotic used in creating the plates. Initially, ampicillin plates were created with anhydrous ampicillin which was far less soluble than ampicillin salt and potentially resulted in inaccurate antibiotic concentrations (17). Despite having a small number of transformants, the two observed colonies may have possessed our plasmid of interest. Results from a restriction enzyme digestion experiment supported this theory (Fig 3A), so we opted to continue with Sanger sequencing rather than perform additional troubleshooting. We did, however, repeat the ligation and transformation reaction using ampicillin salt plates to verify our methodologies, which yielded improved transformation efficiencies (Fig S2). The colonies from the second replicate were not used in downstream experiments.

Following transformation, HindIII restriction enzyme digestion also revealed that our gene of interest was inserted in opposing directions (Fig 3). Subsequently, Sanger sequencing was performed on the plasmids isolated from CFU A (pCR2.1-*wbbL-a*) and CFU B (pCR2.1-*wbbL-β*), in order to verify the sequence of the insert. From this analysis, it was confirmed that both pCR2.1-*wbbL-a* and pCR2.1-*wbbL-β* contained our gene of interest. However, while pCR2.1-*wbbL-a* contained an intact copy of *wbbL,* pCR2.1-*wbbL-β* was found to contain two point mutations within the insert region. These observed single nucleotide polymorphisms may be due to the low fidelity of the Maxima Hot Start Taq Polymerase used for PCR amplification (18). To mitigate this in the future, a higher fidelity Taq Polymerase, such as the Platinum™ Taq DNA polymerase (High Fidelity) by ThermoFisher Scientific may be used instead.

To determine plasmid functionality, pCR2.1-*wbbL-a* and pCR2.1-*wbbL-β* were transformed into BW25113 and JW2203-1 cells, and resistance was evaluated by a stab assay. Cells transformed with pCR2.1-*wbbL-a* exhibited resistance to T4, which is consistent with previous literature. Browning *et al*. found that introduction of an intact *wbbL* gene to the *E. coli* K-12 strain, MG1655, resulted in O-antigen synthesis and resistance to P1 bacteriophage (2). Similarly, in a study by Chiu *et al*., double agar overlay plaque assays revealed resistance against T4 bacteriophage-induced lysis in the *wbbL* restored strain, DFB1655 L9, but not in the *wbbL* disrupted strain MG1655 (8). In contrast, cells transformed with pCR2.1-*wbbL-β* did not exhibit resistance to T4-induced lysis. This may have been due to the presence of the point mutations within the insert or the fact that the *wbbL* insert was in the reverse orientation. Gene expression assays, such as RT-qPCR, may be conducted to determine whether the issue lies at the transcriptional level or, to determine whether WbbL was misfolded due to the point mutations, the structure of the protein may be analysed by nuclear magnetic resonance (NMR) spectroscopy or X-ray crystallography.

Finally, our stab assay results suggest the Δ*ompC* genotype is susceptible to T4. This contrasts with the literature, which has described increased resistance following *ompC* mutation (11, 12). For instance, in a study by Hakkinen *et al*., it was shown that T4 phage resistance developed from cefotaxime antibiotic selection, was associated with *ompC* mutations in *E. coli* cells (11). Additionally, Morgan *et al*. demonstrated that *ompC* deletion corresponded with decreased T4 phage adsorption and T4 phage-induced bacteriolysis (12). However, it is important to note that the qualitative nature of the stab assay may have prevented the detection of the partial or increased resistance noted in the previous studies. Alternatively, another explanation for the discrepancy between our results and the literature may be that the JW2203-1 strain did not contain the *ompC* deletion. This can be verified in future studies through PCR amplification of the *ompC* gene.

Limitations One limitation of our study pertains to the positive control that was used for the HindIII restriction enzyme digestion experiment. Unexpectedly, when run on a 1% agarose gel, the digested control plasmid yielded two bands. The control plasmid was constructed in parallel with our recombinant sample plasmids and contained an unspecified gene fragment that was amplified using primers provided in the TOPO™ TA™ cloning kit. Although the product manual had provided an expected PCR fragment size for the control, the actual sequence was not specified. Presumably, the double bands corresponded to a single cut within the plasmid, and another cut within the insert. However, since we were not provided with the sequence of the insert, no meaningful interpretations could be made. For a more informative positive control, we recommend the use of the linearized vector backbone (pCR2.1) instead.

Another limitation pertains to the use of the stab assay to assess resistance and pCR2.1-*wbbL-a* plasmid functionality. It should be noted that the stab assay is a qualitative test, ideally used to obtain preliminary results and necessitates further quantification and verification through the use of more sophisticated assays. Although we attempted to mitigate variability by having the same person perform all stab assays, the amount of viral titer deposited per stab may have slightly varied and been influenced by the amount of time dipped into the viral stock or the porosity of the wooden stab. As a result, we cannot quantify the degree of resistance, nor compare it between different samples. Furthermore, due to time constraints, we were unable to perform additional replicates so our results could not be statistically analyzed. Nevertheless, for our purposes, the stab assay was sufficient in determining the presence or absence of T4 bacteriophage resistance and provided preliminary support to verify the functionality of our recombinant plasmid.

Conclusions We sought to investigate the combined role of O-antigen and *ompC* deletion together in T4 phage infection. We hypothesized that cells with both mutations will display more resistance than either Δ*ompC* or O-antigen expression independently. To do so, we constructed a *wbbL*-containing recombinant plasmid, pCR2.1-*wbbL-a*. Unexpectedly, we also isolated a second plasmid containing a mutated *wbbL* gene inserted in the opposite direction, which we denoted as pCR2.1-*wbbL-β*. These plasmids were subsequently transformed into two cell lines, BW25113, a wild-type strain, and JW2203-1, an Δ*ompC* strain. Resistance to T4 bacteriophage was then qualitatively evaluated using stab assays. Cells transformed with pCR2.1-*wbbL-a* displayed no lawn of clearance indicating resistance to T4 bacteriophage, suggesting both the expression of O-antigen and functionality of the plasmid. Overall, the role of Δ*ompC* and O-antigen together are inconclusive due to the apparent susceptibility of Δ*ompC* cells to T4 bacteriophage, which contradicts previous literature. Nevertheless, this work has resulted in a selection of *E. coli* strains that if phenotypically verified, will provide a range of *E. coli* phenotypes available for use in future studies.

Future Directions Although we were able to construct a plasmid to restore T4-phage resistance in BW25113 and JW2203-1 (Δ*ompC*) *E. coli* strains, we were unable to quantify the degree of resistance to effectively compare the conditions. Future studies can focus on quantitative methods, such as a double agar overlay plaque assay or a lytic assay, to measure, quantify, and more accurately conclude the bacteriophage resistance of our plasmid transformed strains. Because these assays are more sensitive, they may reveal a difference in resistance between the empty-vector transformed Δ*ompC* (JW2203-1) and wild-type strain (BW25113), which was not seen in the stab assay but suggested in the literature (11, 12).

Furthermore, due to time constraints, the expression of O-antigen was not explicitly confirmed in pCR2.1-*wbbL-a* transformed *E. coli* cells; rather, O-antigen expression was suggested by the restoration of T4-phage resistance. To verify this, the LPS of transformed strains can be isolated and analyzed using SDS-PAGE. If O-antigen is present, subsequent silver staining should reveal a smear around 50-60kDa (8). Alternatively, agglutination reactions that employ antisera against O-antigen or genetics-based PCR assays that target unique gene sequences within O-antigen gene clusters, can also be used to detect the presence of O-antigen (19). A possible confounding factor for longer assays would be the loss of O-antigen expression overtime following transformation. To verify the stability of O-antigen

expression, an analysis of the expression profiles of O-antigen over extended T4-incubation may be conducted.

After phenotypic verification, pCR2.1-*wbbL-a* can be used to transform other knock-out *E. coli* strains from the Keio Collection (ΔTsx, ΔOmpA, ΔOmpF, ΔFadL) to explore the relationship between various T-even bacteriophage cell surface receptors and O-antigen expression (20). Furthermore, pCR2.1-*wbbL-a* can also be used to study the mechanism of T1 bacteriophage adsorption, since it can use O-antigen as a primary cell-surface receptor (20).

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CONTRIBUTIONS

All participants participated equally in investing time at the UBC Microbiology & Immunology Laboratory and in writing the manuscript. In the writing of the manuscript, C.Q. and E.C. collaborated on the introduction. E.C. completed the materials and methods. C.Q, D.S., and E.C. collaborated in the writing of the results, discussion, and conclusion. E.C. and D.S. completed the limitations and future directions sections. C.Q. formatted the supplemental material. All members collaborated on the writing of the title, abstract, figures and editing of the manuscript.

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