

# Developing the Antisense Silencing Model for the Investigation of the Mechanism of Resistance of *Escherichia coli* DFB1655 L9 to T4 Bacteriophage

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**SUMMARY** O antigen consists of multiple repeating polysaccharide units found on lipopolysaccharide in some Gram-negative bacteria, including *Escherichia coli*. It is considered to be a virulence factor as well as a point of interaction with certain bacteriophages. The *E. coli* strain MG1655 has an insertion of an IS5 element within *wbbL*, preventing WbbL expression, thus interrupting the O antigen synthesis pathway. DFB1655 L9 is generated from the isogenic strain MG1655 to restore functional *wbbL* via a single crossover homologous recombination. MG1655 is susceptible to T4 bacteriophage infection whereas DFB1655 L9 is resistant, however the mechanism of DFB1655 L9 resistance is unknown. In this study, we hypothesize that the insertion of *wbbL* and the subsequent expression of O16 antigen in DFB1655 L9 confer resistance to T4 infection. We confirmed that DFB1655 L9 is resistant to T4 infection up to a multiplicity of infection of 20. We also designed pCODA-*wbbL*(a) for antisense RNA silencing of *wbbL* by cloning the antisense ribosome binding site of *wbbL* into pHN678 and transforming into DFB1655 L9.

## INTRODUCTION

The O antigen is a repetitive oligosaccharide unit found in the lipopolysaccharide (LPS) on most Gram-negative bacterial outer membrane, including *Escherichia coli* (1). A single O unit can be made up of 2-8 residues of different sugars linked together which leads to the high variability of serotypes (1). Due to laboratory culturing conditions, many strains of *E. coli* have acquired mutations to help them adapt to the artificial environment; among which *E. coli* strain K-12 lost the ability to produce O antigen due to an IS5 insertion in *wbbL*, the last gene in the *rfb* cluster (2, 3). Hence, the MG1655 strain lacks the expression of O antigen on the outer membrane. *wbbL* encodes a rhamnosyltransferase involved in the production of O16 serotype LPS by transferring a L-rhamnose to the synthesizing polysaccharide chain (4). From MG1655, Browning *et al.* created *E. coli* DFB1655 L9, which rescued WbbL expression through a single crossover homologous recombination, giving DFB1655 L9 an intact *rfb* cluster (5). DFB1655 L9 is resistant to infection by bacteriophage P1, T4, and T7, as well as complement killing; whereas MG1655 is susceptible to all of the above (4, 6, 7). However, the mechanism of the resistance of DFB1655 L9 to T4 remains undetermined.

Bacteriophages are viruses that infect bacteria, and there are seven types of phages (T1-T7) originally described by Demerec and Fano (6). Bacteriophage T4 infects *E. coli* K-12 (7). It consists of a tail (tube-like structure) with a contractile sheath, six tail fibres, and an icosahedral head enclosing a 169-kbp double stranded DNA genome (7). T4 attaches to the LPS and outer membrane protein C (OmpC) on the surface of *E. coli* through the tail fibres and infects *E. coli* by releasing the viral DNA into the host (8, 9). Another mechanism for attachment involves using only LPS with a glucose residue exposed on the end as a receptor (2). Both mechanisms require the presence of LPS on the outer membrane surface of *E. coli* for T4 infection.

MG1655 is a strain of *E. coli* K-12 that is susceptible to T4 infection, while DFB1655 L9, having *wbbL* rescued, is resistant to T4 infection. Previous studies show that the expression of O16 antigen in DFB1655 L9 is sufficient to increase resistance to T4 infection (1-3, 10). One proposed mechanism for the resistance of DFB1655 L9 to T4 infection is a steric hindrance model in which the O antigen prevents interaction between the phage tail

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fibres and LPS, interrupting attachment of T4 (5). However, this explanation is not further investigated.

Since DFB1655 L9 only differs genomically from MG1655 by the restored *wbbL*, which is involved in O antigen synthesis, it is important to look into the role of *wbbL* with respect to T4 infection resistance. In this study, we aim to investigate whether the silencing of *wbbL* translation in DFB1655 L9 could reduce its resistance to T4. Conditional gene silencing in *E. coli* can be achieved by using an antisense RNA (asRNA) to the ribosome binding site (RBS) of *wbbL*. When transcribed, the asRNA will hybridize the RBS of *wbbL*, preventing recognition by ribosome and translation of *WbbL* (11). In particular, asRNA with paired-termini (PT) carrying flanking inverted repeats can create paired double stranded RNA termini when transcribed (11). This helps stabilize the asRNA and increase the efficacy compared to single stranded asRNA (11). The plasmid, pHN678, contains adjacent restriction enzyme cut sites of *XhoI* and *NcoI* which allow the ligation of an antisense DNA fragment (11). The *lac* operator and repressor genes allow asRNA transcription to be induced by isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) (8, 9, 11).

By using the antisense RNA, we aimed to further investigate the mechanism of resistance of DFB1655 L9 to T4 infection. We hypothesize that the insertion of *wbbL* and the subsequent expression of O16 antigen in DFB1655 L9 is sufficient to confer resistance to T4 infection. Although it was not explored, it could be possible that the rescue of *wbbL* in DFB1655 L9 impacted other mechanisms in addition to O antigen synthesis in the cells which render them resistant to T4 infection. To test our hypothesis, we produced a plasmid, pCODA-*wbbL*(a), containing a PT asRNA targeting the RBS of *wbbL* to silence the expression of O antigen in DFB1655 L9. After successfully transforming the recombinant plasmid into both MG1665 and DFB1655 L9 and infecting them with T4, initial experiments indicated that the DFB1655 L9 with pCODA-*wbbL*(a) remained resistant to T4 bacteriophage. We also tested the steric hindrance model by generating a lysis curve of MG1665 and DFB1655 L9 at varying multiplicity of infection (MOI) to test whether a high concentration of phage could overcome the resistance of DFB1655 L9 to T4. We found that DFB1655 L9 remained resistant to T4 even at high MOI.

## METHODS AND MATERIALS

**Bacterial strains used.** *E. coli* K-12 substrains MG1655 and DFB1655 L9 were gifts from Dr. Douglas F. Browning from University of Birmingham. MG1655 had *wbbL* interrupted with an IS5 insertion element, and was unable to synthesize O16 antigen. DFB1655 L9 had *wbbL* rescued via single crossover recombination of a functional *wbbL* gene obtained from *E. coli* K-12 strain WG1 (5). DFB1655 L9 was able to synthesize O16 antigen (5). MG1655 was propagated on 1.5% Luria-Bertani (LB) agar. DFB1655 L9 was propagated on 1.5% LB agar supplemented with 50  $\mu$ l/ml kanamycin. Plates were incubated at 37°C overnight. Isolated colonies of MG1655 and DFB1655 L9 were streaked onto LB slants and LB slants with kanamycin, respectively, incubated at 37°C overnight and stored at 4°C subsequently.

**TABLE 1 Primer sequences for PCR amplification.** F and R denotes forward and reverse, respectively.

Gene	Sequence (5'-3')	Size (bp)
<i>wbbL</i>	F: CCCGAATTCATATGGTATATATAATAATCGTTTCCC	1994 (MG1655)
	R: CCCAAGCTTCTCGAGTTACGGGTGAAAACTGATGAAATTC	799 (DFB1655 L9)
<i>gp23</i>	F: GCCATTACTGGAAGGTGAAGG	398
	R: TTGGGTGGAATGCTTCTTAG	
<i><math>\beta</math>-lactamase</i>	F: CTACATACCTCGCTCTGCTAATC	188
	R: CACGCTGTAGGTATCTCAGTTC	

**Amplification of T4.** MG1655 was cultured in 50 ml LB broth at 37°C, on a rotary shaker at 150 RPM until an OD<sub>600</sub> of 0.33 was reached. 100 µl of 7.1x10<sup>9</sup> plaque forming units (PFU)/ml T4 stock was added and the flask was incubated at 37°C on a rotary shaker at 150 RPM for roughly 4 hours. The culture was centrifuged at 4000 RPM for 5 minutes to remove bacterial debris (Beckman Coulter Avanti J-30I). The bacteria were lysed with 150 µl of chloroform and left at 4°C overnight to ensure all bacteria were lysed. The top aqueous layer was removed and filter-sterilized with a 0.45 µm filter.

**Double agar overlay plaque assay.** Adapted from Wachtel *et al.* (10). 1.5% LB agar was used for the underlay, 0.4% LB agar was used for the overlay and both layers were supplemented with 1 mM CaCl<sub>2</sub>. 100 µl of the appropriate dilution of T4 (10<sup>-5</sup>, 10<sup>-6</sup>, 10<sup>-7</sup>, 10<sup>-8</sup>, 10<sup>-9</sup>) and 100 µl of an overnight culture of MG1655 were added to the overlay before spreading the mixture on top of the solid underlay and allowed to solidify. Viral titer was determined to be 4.45x10<sup>9</sup> PFU/ml.

**PCR verification of strains.** Colony PCR was performed on MG1655 and DFB1655 L9. A small amount of bacterial colony was transferred into PCR tubes using a P200 pipette tip. PCR amplification was performed using Invitrogen™ Platinum™ *Taq* DNA Polymerase (Cat#10966026) following manufacturer's protocol with the Bio-Rad T100™ Thermal Cycler. For MG1655 and DFB1655 L9, *wbbL* was amplified; whereas for T4, *gp23* encoding the major capsid protein was amplified (7). Primer sequences for *wbbL* were obtained from Browning *et al.* (1). T4 specific *gp23* primers were obtained from previous studies (1, 3). Amplification of beta-lactamase gene in pUC19 was used as positive control. The touchdown PCR cycle used for *wbbL* is 5 min denaturation at 95°C followed by 6 cycles of 30 sec at 95°C, 45 sec at 61°C decreasing 1°C per cycle to 53°C on last cycle, then 2.5 min 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. For T4, initial denaturation was set at 95°C for 2-minutes, followed by 30 cycles of 45 sec at 95°C, 30 sec at 51°C, 30 sec at 75°C (9). All PCR products were run on 1% agarose gel in 1x TAE buffer at 100V for 1.5 hours. The gel was visualized with SYBR™ Safe (ThermoFisher) and TrackIt 1 Kb Plus DNA ladder (ThermoFisher) was used. Resolution of the PCR products of MG1655, DFB1655 L9 and T4 on a 1% agarose gel displayed bands at approximately 2000 bp, 800 bp and 400 bp respectively (Fig. 2).

**Comparison of T4 infectivity with varying MOI on MG1655 and DFB1655 L9.** Overnight cultures of MG1655 and DFB1655 L9 were prepared in LB broth. The overnight culture was diluted 1:100 into 15 ml LB culture, and grown at 37°C, shaken at 150 RPM until log phase was reached. OD<sub>600</sub> was determined using the Pharmacia Biotech Ultrospec 3000 spectrophotometer, and a conversion factor of 1 OD<sub>600</sub> = 8x10<sup>8</sup> cells/ml was used. Using a clear flat-bottomed, 96 well plate, 75 µl of bacteria were used and MOI is varied by varying T4 volume. MOI of 0, 1, 5, 10, 20 were tested, along with a no inoculant LB

**TABLE 2 Primer sequences for antisense construction.** F and R denotes forward and reverse, respectively. Lower case denotes restriction sites and upper case denotes insert sequence.

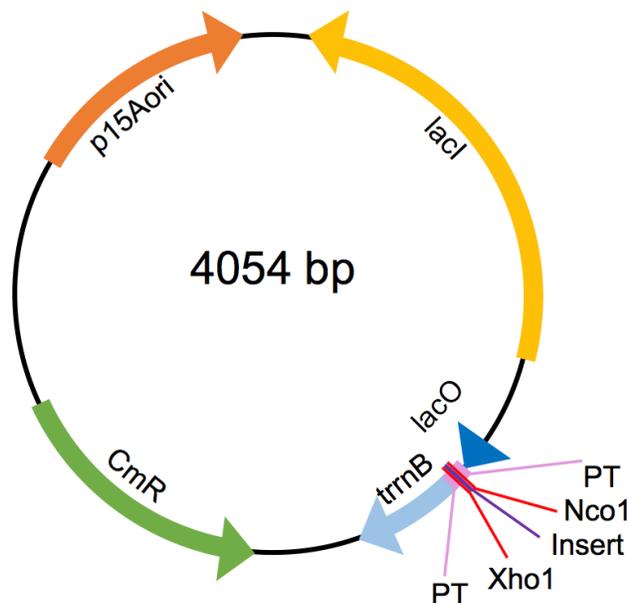
Name	Sequence (5'-3')
Antisense Sequence	F: catggTATACCATTTCAATGTTCTTCAGTAATAAAAATTAAGTTCATCAAAc R: tcgagTTTGATGAACTAGTTAATTTTATTACTGAAGAACATTGAAATGGTATAc
Directionality Control	F: catggAAACTACTTGATCAATTAATAAATAATGACTTCTTGTAACCTTACCATATc R: tcgagATATGGTAAAGTTACAAGAAGTCATTATTTTAATTGATCAAGTAGTTTc

control. LB was supplemented to a final volume of 250  $\mu$ l was used for each well. A total of eight technical replicates were performed for each condition. The incubation growth curve was done at 37°C with shaking for 20 hours (BioTek Synergy™ H1). OD<sub>600</sub> was measured in 10 minutes interval.

**Designing *wbbL* asRNA.** The design of asRNA was adapted from Ji *et al.* (9). The sequence complementary to *wbbL* transcript was obtained from NCBI accession number U00096.3. The oligonucleotides with the antisense *wbbL* RBS sequence were synthesized as forward and reverse primers. The designed primer contained eight base pairs downstream and forty base pairs upstream with respect to the start codon of *wbbL* (Table 2). A directionality control sequence was also designed, in addition to the antisense, to ensure the correct orientation of antisense transcript. NcoI and XhoI overhangs were included in the primer sequence, so that the primer would not require restriction enzyme digestion after annealing. The primers were ordered from Integrated DNA Technologies (IDT) and were diluted with autoclaved distilled water to 100  $\mu$ M stock concentration. The primer stocks were stored at -20°C.

**Annealing antisense primers.** The primers were annealed by adding 20  $\mu$ l each of forward and reverse primer stocks and heated to 95°C for 2 minutes. The primer mixture was slowly cooled down in the heat block until it reached room temperature. The annealed primers were treated with T4 polynucleotide kinase for phosphorylation of the 5' end. The annealed primers were stored at -20°C.

**Amplification of pHN678.** Vector pHN678 was a gift from Dr. Liam Good from Department of Pathology and Infectious Diseases, Royal Veterinary College. pHN678 contains NcoI and XhoI restriction sites flanked by PT to stabilize the asRNA as shown in Fig. 1 (11). The expression of asRNA is controlled by *lacO*, and can be induced with IPTG. DH5 $\alpha$  carrying pHN678 were grown overnight at 37°C on rotary shaker in 2x 5 ml of LB supplemented with chloramphenicol. Chloramphenicol was used at 25  $\mu$ g/ml final concentration unless otherwise stated. Mini-Prep was performed with Biobasic EZ-10 Spin Column Plasmid DNA Minipreps Kit (Cat# BS614) according the manufacturer's protocol for low copy number plasmids. Final elution was in 50  $\mu$ l of autoclaved distilled water. The final yield was determined with Nanodrop 2000c (ThermoFisher).

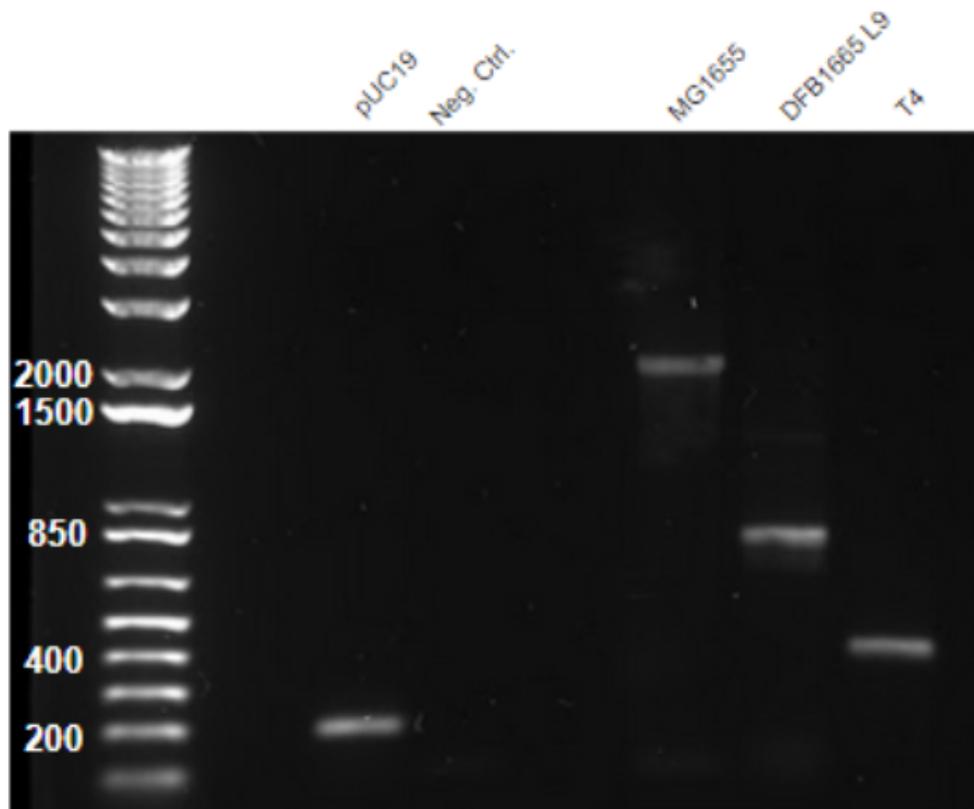


**FIG. 1** Vector pCODA-wbbL(a)/(d) map. Plasmid map of pCODA-wbbL(a)/(d) created from a plasmid editor (ApE). Insert with antisense sequence was termed pCODA-wbbL(a) and insert with directionality control sequences is termed pCODA-wbbL(d) (Table 2).

**NcoI and XhoI digestion of pHN678.** Restriction enzyme digestion was performed with NcoI-HF and XhoI according to manufacturer's protocol (New England BioLabs, Cat. #R3193S and Cat. #R0146S respectively). Double-digestion was done simultaneously in the same tube. The digested products were run on 1% agarose gel at 100 V for 1.5 hours. Gel products were excised and purified with GeneJET Gel extraction kit by ThermoFisher (Cat# K0691). Samples were eluted in 50  $\mu$ l water and stored at -20°C.

**Ligation of *wbbL* antisense and directionality control into pHN678.** Ligation was done using Invitrogen T4 DNA Ligase (Ref 15224-017), following the manufacturer's protocol for sticky end ligation. 1  $\mu$ l of ligase was used for reaction. Refer to Table 2 for insert sequences. Insert to plasmid ratio of 3:1 was used. Molar mass of annealed primers was determined by the addition of molar mass of forward and reverse primer. Dilution of annealed primers was carried out with autoclaved distilled water. Ligation reaction was kept at room temperature for 1 hour. Ligation products were diluted 5-fold to 100  $\mu$ l prior to transformation, as suggested by the protocol. Isolated colonies were picked and grown in 2 tubes of 5 ml of LB with chloramphenicol overnight on a rotary shaker at 150 RPM at 37°C. Mini-Prep was performed as previously described in amplification of pHN678.

**Preparing competent *E. coli* DH5 $\alpha$ , MG1655, and DFB1655 L9.** An isolated bacterial colony was added to 2 ml of LB broth and incubated at 37°C on a rotary shaker at 150 RPM for 18 hours. Overnight culture was subcultured 1:100 in 50 ml of LB broth and incubated at 37°C on a 175 RPM shaker until log phase. Culture was centrifuged at 4000 RPM for 10 minutes at 4°C (Beckman Coulter Avanti J-30I). Supernatant was discarded and each pellet was resuspended in 10 ml of ice-cold 0.1M CaCl<sub>2</sub> and incubated on ice for 30 minutes. After incubation on ice, the resuspension was centrifuged at 4000 RPM for 10 minutes 4°C and the supernatant was discarded. Pellets were resuspended in 3.5ml 0.1M CaCl<sub>2</sub> and



**FIG. 2** Verification of *Escherichia coli* K-12 strain MG1655, DFB1655 L9, and T4. Touchdown PCR was performed for MG1655, DFB1655 L9, and pUC19 (positive control). The 1994 bp band in MG1655 represented *wbbL* with IS5 insertion element. The 799 bp band in DFB1655 L9 represented *wbbL*. The 398 bp band in T4 represented the T4 specific *gp23*.

1.5ml of 50% glycerol solution. The competent cells were divided into 100  $\mu$ l aliquots and stored at  $-70^{\circ}\text{C}$ .

**Propagation of pCODA-wbbL(a) and pCODA-wbbL(d) in DH5 $\alpha$ .** 20  $\mu$ l of ligation products were incubated with 100  $\mu$ l of competent DH5 $\alpha$  on ice for 30 min. The reaction was heat shocked at  $42^{\circ}\text{C}$  for 30 seconds, followed by incubation on ice for 2 minutes. The transformed cells were rescued with 900  $\mu$ l of warmed LB broth and incubated at  $37^{\circ}\text{C}$  on a shaker for 1 hour. 100  $\mu$ l of transformed cells were plated on LB plates with chloramphenicol and incubated overnight at  $37^{\circ}\text{C}$ . Inserts was confirmed with Sanger sequencing. pHN678 with insert from antisense sequence is termed pCODA-wbbL(a) and pHN678 with insert from directionality control is termed pCODA-wbbL(d) (Table 2). Experiments performed with both pCODA-wbbL(a) and pCODA-wbbL(d) used the abbreviation of pCODA-wbbL(a)/(d).

**Transformation of pCODA-wbbL(a) and pCODA-wbbL(d) into MG1655 and DFB1655 L9.** Colonies with confirmed inserts were grown in 2 tubes of 5 ml of LB with chloramphenicol overnight on a rotary shaker at 150 RPM at  $37^{\circ}\text{C}$ . Mini-Prep was performed as previously described in amplification of pHN678. 1  $\mu$ l of pCODA-wbbL(a)/(d) were incubated with 100  $\mu$ l of competent MG1655 or DFB1655 L9 on ice for 30 minutes. The reaction was heat shocked at  $42^{\circ}\text{C}$  for 30 seconds, followed by incubation on ice for 2 minutes. The transformed cells were rescued with 900  $\mu$ l of warmed LB broth and incubated at  $37^{\circ}\text{C}$  on a shaker for 1 hour. 100  $\mu$ l of transformed cells were plated. MG1655 was plated on LB supplemented with chloramphenicol while DFB1655 L9 was plated on LB supplemented with kanamycin and chloramphenicol. The plates were incubated inverted overnight at  $37^{\circ}\text{C}$ .

**Growth curve of MG1655 and DFB1655 L9 transformed with pCODA-wbbL(a) and pCODA-wbbL(d).** Colonies of successful transformants were picked and grown in 2ml LB supplemented with 1 mM IPTG and appropriate antibiotics. MG1655 was cultured with LB, while MG1655 with pCODA-wbbL(a)/(d) was cultured with LB and chloramphenicol. DFB1655 L9 was cultured with LB and kanamycin, while DFB1655 L9 with pCODA-wbbL(a)/(d) was cultured with LB, kanamycin and chloramphenicol. IPTG was used to induce transcription of asRNA. The culture was grown to log phase. 75  $\mu$ l of bacteria culture was used across all conditions, and MOI of 3 was achieved by varying phage volume. LB media supplemented with 1 mM IPTG and appropriate antibiotics was added to all conditions for a final volume of 150  $\mu$ l. A total of eight technical replicates were completed for each condition. LB with T4 stock was used as blank control. The incubation growth curve was measured at  $37^{\circ}\text{C}$  with shaking for 5 hours (BioTek Synergy<sup>TM</sup> H1).  $\text{OD}_{600}$  was measured in 5 minutes intervals.

## RESULTS

**DFB1655 L9 remains resistant to T4 infection up to MOI of 20.** Previous studies show that DFB1655 L9 is resistant to T4 bacteriophage infection; having been observed through viral lysis assays in which cultures of the bacteria retain a high optical density despite an incubation with the virus (2). T4 needs to interact with receptors to gain entry to cell. If the resistance to T4 in DFB1655 L9 is due to steric hindrance imposed by the restored O16 antigen production, an oversaturation of T4 may overcome the decreased accessibility to the viral receptors. Since viral particles randomly interacts with receptors, the chance of collision can be described by the Poisson distribution (12). According to the Poisson distribution, with a MOI of 5 or greater, more than 99% of cells should have been infected by the virus (12). To test whether a high MOI of T4 could overcome the resistance exhibited by DFB1655 L9, DFB1655 L9 was subjected to MOI ranging from 0 to 20 in coinoculation with T4 at  $37^{\circ}\text{C}$  for 20 hours. MG1655 subjected to varying MOI was used as control for T4 infectivity and LB media alone was used to verify no contamination in the media that could account for rising of  $\text{OD}_{600}$  in DFB1655 L9. DFB1655 L9 remained resistant to T4, even at a MOI of 20. MG1655 was susceptible to T4 lysis at all tested MOIs

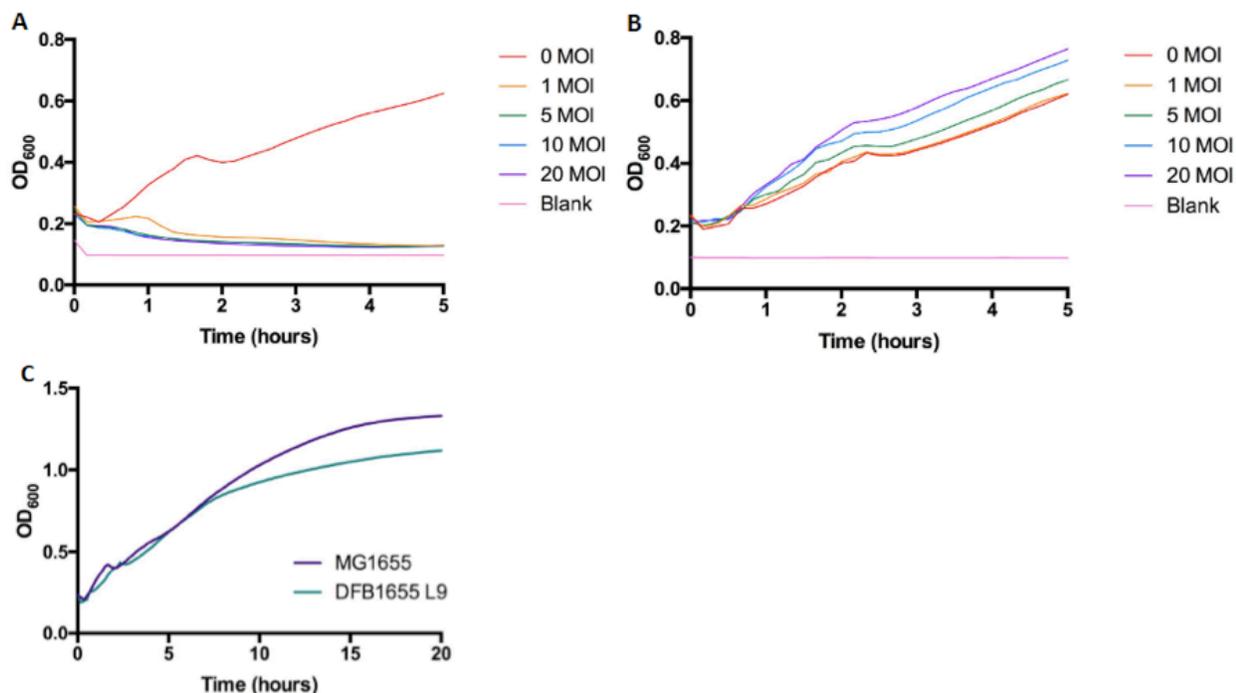
above zero. Above a MOI of 5, there were no differences in MG1655 lysis between various MOIs due to oversaturation of T4. There was no contamination in the LB media used, as indicated by the blank control (Fig. 3B). The OD<sub>600</sub> of DFB1655 L9 did not decrease even at 20 MOI.

**MOI-dependent increase in DFB1655 L9 growth yield.** While performing the lysis curve analysis of DFB1655 L9 with T4, an interesting trend was observed. With an increase in MOI, DFB1655 L9 appeared to grow to a higher OD as shown in Fig. 3B. The effect of the MOI-dependent increase in cell density was observed over the 20-hour incubation (Fig. S1). T4 was cultured with log phase MG1655 and chloroform was added to ensure complete lysis of bacteria. Since MOI was achieved by varying phage stock volume and not the amount of bacteria seeds, perhaps there are some growth factors present in the phage medium. Since this was a single biological replicate, the experiment should be repeated to confirm this phenomenon. The trend could be due to mechanical or technical errors or inconsistencies.

**MG1655 reaches higher cell density than DFB1655 L9.** Non-infected MG1655 and DFB1655 L9 were used as control for cell viability. MG1655 reached OD<sub>600</sub> of 1.33 after growing for 20 hours and DFB1655 L9 reached an OD<sub>600</sub> of 1.12 in the same amount of time (Fig. 3C). The growth difference was also observed in colony morphology, independent to kanamycin treatment, when subjected to the same duration of incubation (Fig. S3). DFB1655 L9 grew slower and plateaued earlier than MG1655.

#### Generation of pCODA-wbbL(a) used in asRNA silencing of *wbbL* inducible by IPTG.

A plasmid, pCODA-wbbL(a) was produced which contained PT regions flanking the antisense *wbbL* RBS. Following previous methods by Ji *et al.*, the targeted area for asRNA knockdown of the *wbbL* was selected to be approximately 50 base pairs upstream of the start codon found on the intact gene in DFB1655 L9. Since *wbbL* is expressed on a polycistronic mRNA, asRNA binding to upstream of start codon prevented the ribosome

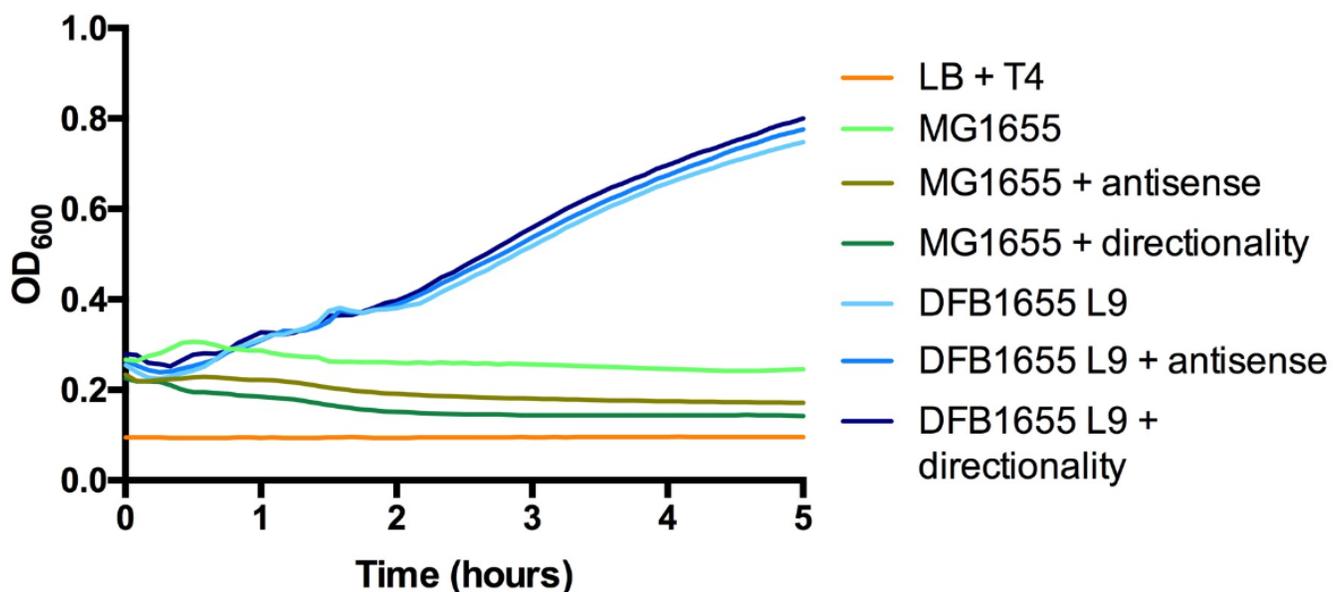


**FIG. 3** Growth curve of *Escherichia coli* K-12 substrain MG1655 and DFB1655 L9 show inherent differences. (A) Average growth curve of MG1655 with varying MOI of T4. Blank represented LB media alone. All conditions were performed with eight replicates. MG1655 was susceptible to T4 lysis. (B) Average growth curve of DFB1655 L9 with varying MOI of T4. Blank represents LB media alone. DFB1655 L9 remained resistant to T4 lysis. (C) Comparison of average growth curve of MG1655 and DFB1655 L9. MG1655 reached higher an OD<sub>600</sub> than DFB1655 L9 in LB media.

from docking, and therefore decreasing the translation of WbbL. The transcribed mRNA will form a hairpin structure as the PT complements, with the asRNA sequence at the tip of the loop. The subsequent asRNA sequence was generated via antisense complementary base pairing with the sequence found in the genome (Table 2). Each end of the insert was modified to contain specific bases that would pair with the NcoI and XhoI cut sites, allowing for proper integration of the insert between the two restriction endonuclease sites in pHN678 (13). The vector pHN678 contains a *lac* operator and repressor, so that the *wbbL* RBS asRNA transcription could be induced by IPTG, which could be taken advantage of in different experimental designs in studying the role of *wbbL* in DFB1655 L9. For example, the asRNA production could be inactivated or induced by IPTG at different time points. Finally, a directionality control, pCODA-wbbL(d), was created in order to confirm the insert was ligated in the correct orientation (Fig. 2).

**Sequencing of pCODA-wbbL(a)/(d) confirms presence of *wbbL* asRNA.** In order to verify whether or not pCODA-wbbL(a) contains the intended *wbbL* RBS asRNA sequence, the plasmid was sequenced with a primer annealing close to the cloning site. The hairpin structure posed to be difficult in Sanger sequencing. Confidence of sequencing results, interpreted as fluorescent peak heights, dropped as the first PT sequence was encountered (data not shown). The hairpin structure may have affected the processivity of the polymerase. Despite the low sequence confidence, reproducible sequencing of inserts was possible. The sequencing results for the pCODA-wbbL(a) amplified in DH5 $\alpha$  showed that the *wbbL* asRNA was inserted in the multiple cloning site. The directionality control confirmed that the asRNA sequence was designed in the correct orientation. It is also worth noting that sequencing of what we presumed was the empty pHN678 vector revealed a foreign sequence aligning with a common plasmid sequence between XhoI and NcoI cut sites. This was unexpected since provided pHN678 sequence did not indicate any insert between the cut sites, and that the cut sites should have been directly adjacent to each other (8).

**Silencing of *wbbL* did not render DFB1655 L9 susceptible to T4 infection.** To investigate whether WbbL is necessary in T4 resistance in DFB1655 L9, *wbbL* was silenced at the translational level with asRNA. MG1655 and DFB1655 L9 were transformed with



**FIG. 4** Silencing of *wbbL* in DFB1655 L9 did not reduce T4 resistance. Antisense denotes pCODA-wbbL(a) and directionality denotes pCODA-wbbL(d). Average growth curve of eight technical replicates are shown for each condition. Chloramphenicol is used for MG1655 carrying pCODA-wbbL(a)/(d). Kanamycin was used for DFB1655 L9, and chloramphenicol and kanamycin were used for DFB1655 L9 carrying pCODA-wbbL(a)/(d). MOI of 3 was used for all conditions. IPTG was added to all conditions except for LB+T4 blank control.

pCODA-wbbL(a)/(d). Antibiotics selection pressure was kept throughout the experiment, from transformation to growth curve assay. IPTG induction started when MG1655 and DFB1655 L9 were grown in culture prior to the growth curve assay, and was continued throughout to maintain asRNA induction. MG1655 served as control for T4 infectivity. pCODA-wbbL(d) served as directionality control for *wbbL* asRNA. MG1655 transformed with pCODA-wbbL(a) served as control of any side effects of *wbbL* asRNA. MOI of 3 was chosen for all conditions. LB with T4 was used as control for contamination. IPTG induction in DFB1655 L9 carrying pCODA-wbbL(a) did not render DFB1655 L9 susceptible to T4 lysis at MOI of 3. There were no differences between DFB1655 L9 with pCODA-wbbL(a)/(d), indicating the directionality of our insert did not play a role. The next step is to confirm the successful decrease in WbbL with western blot. If silencing of *wbbL* transcript was successful, then WbbL is not necessary for T4 resistance in DFB1655 L9.

## DISCUSSION

It has been well described that MG1655 is susceptible to T4 infection whereas DFB1655 L9 is resistant; however, the mechanism of this resistance is yet to be understood (1-3, 10). If DFB1655 L9 is resistant to T4 due to steric hindrance, increasing the MOI should increase the number of viral binding events, potentially causing an increase in susceptibility of DFB1655 L9 to T4 infection. It has also yet to be confirmed whether the silencing of WbbL will increase susceptibility of DFB1655 L9.

**Single *wbbL* amplicon in DFB1655 L9 despite two genomic *wbbL* copies.** To verify the *E. coli* strains MG1655 and DFB1655 L9, colony PCR was performed. In contrast to previous study performed by Chiu *et al.*, a single prominent band was observed for both strains after gel electrophoresis (3). In Fig. 2, touchdown PCR of DFB1655 L9 only show one band approximately 800 bps in length, indicating the uninterrupted *wbbL*. In MG1655, there is only one *wbbL* sites to amplify from. In DFB1655 L9, there are two *wbbL* sites the primers can anneal to, since the primer sequences bind within *wbbL*. DFB1655 L9 is constructed by a single crossover homologous recombination with the suicide vector pJP5603 (1). Both uninterrupted and interrupted *wbbL* should be observed in DFB1655 L9, as shown before by Chiu *et al.* (3). The reason why a single band is observed for DFB1655 L9 can be explained by the primer design originally from Browning *et al.* (1). The primers used to amplify *wbbL* contain built-in restriction sites for cloning purposes. The reverse primer for *wbbL* contains restriction sites for HindIII and XhoI. It has a higher predicted melting temperature at the functional *wbbL* compared to the interrupted *wbbL*, at 59°C versus 54°C, respectively. This is due to addition of annealing to adjacent Sall sites on pJP5603. The touchdown PCR annealing starts at 61°C; therefore, a higher amount of reverse primers will bind to the functional *wbbL* region, creating more copies at the start of amplification. As for the forward *wbbL* primers, the predicted melting temperature is lower, at 52°C when including the EcoRI restriction site, and 44°C with just the *wbbL* sequences. Although forward *wbbL* will bind more preferentially to the interrupted *wbbL* region, due to addition of annealing to EcoRI sites, the melting temperature is much lower. By the time the melting temperature is more favorable, more copies of amplicons of functional *wbbL* is present in the mixture. Therefore, there is higher chance of encountering those amplicons than the genomic fragments. The *E. coli* strains were verified using touchdown PCR and gel electrophoresis.

**High MOI did not overcome resistance to T4 imposed by DFB1655 L9.** One proposed mechanism for T4 resistance in DFB1655 L9 is the steric hindrance model, in which the O antigen on the surface of the bacteria prevents interaction between the bacteriophage tail fibres and receptors on DFB1655 L9 (5). To investigate whether T4 resistance in DFB1655 L9 was due to steric hindrance, DFB1655 L9 was incubated with T4 at high MOIs up to 20. It was found that DFB1655 L9 remained resistant to T4 despite the increasing MOI. If the explanation to T4 resistance in DFB1655 L9 is to be due to steric effects, the use of a high MOI should be able to bypass the O antigens as some phage would be able to gain access to the receptors at a very high concentration. However, we observed no susceptibility to T4 in DFB1655 at an MOI of 20. Moreover, we observe that DFB1655 L9 proliferated slightly

better with an increase in MOI. This might be an artifact due to growth factors present in the phage stock medium. Further experiments are required to confirm this phenomenon. With no increase in susceptibility of DFB1655 L9 to T4 infection, the results did not concur with the steric hindrance model, indicating that there can be other mechanisms involved which confer T4 resistance in DFB1655 L9.

**WbbL is not necessary for T4 resistance in DFB1655 L9.** Although data from silencing of *wbbL* translation did not support our hypothesis, it aligns with our MOI experiment. In the MOI experiment, DFB1655 L9 was subjected to MOI of 20, an oversaturation of T4. Assuming that the oversaturation is sufficient to allow T4 to interact with its receptors on DFB1655 L9, then steric hindrance imposed by O antigen is not sufficient to explain the failure of T4 infection. Further, DFB1655 L9 and MG1655 show differences in growth curve. DFB1655 L9 plateaus earlier than MG1655 and possesses a smaller colony size on LB agar plate regardless of kanamycin treatment. Colony size limitation is primarily determined by microbial catabolites diffused into medium and not nutrient depletion (13). This indicates differences between MG1655 and DFB1655 L9 other than the energy consumption by O antigen synthesis.

If the stationary phase is due to nutrient depletion instead of inhibitory compounds, it can still be suggested that there are inherent metabolic differences between DFB1655 L9 and MG1655. DFB1655 L9 plateaus earlier than MG1655 and both received similar amount of nutrients. This means the portion carbon source in media used by MG1655 to make cell mass can be used by DFB1655 L9 for energy consumption. The single crossover incorporation of suicide vector pJP5603, as well as expression of *wbbL* and subsequent synthesis of O antigen poses an extra energetic cost for DFB1655 L9. The extra energetic cost is not likely to be accountable for the reduction in DFB1655 L9 cell mass seen in Fig. 3C, suggesting additional accounted metabolic processes differences between MG1655 and DFB1655 L9. Using OD<sub>600</sub> values at the end of 20 hour run from Fig. 3C, conversion factor of 1 OD<sub>600</sub> = 8x10<sup>8</sup> CFU/ml, 250 µl culture volume per well, it is calculated that DFB1655 L9 possess roughly 4.2x10<sup>7</sup> cells more than MG1655. *E. coli* dry weight per cell is 2.8x10<sup>-13</sup> g and 1 mol of ATP can produce 10 g of dried bacteria cell mass (14, 15). Therefore, DFB1655 L9 has roughly 3.16x10<sup>9</sup> extra ATP per cell compared to MG1655, which can be used for other cellular processes. Energy available from ATP is defined as hydrolysis of ATP to ADP herein. There is an energetic cost in maintaining genome incorporation of suicide vector pJP5603, which carries a functional *wbbL*, as well as RNA transcripts and protein expression of WbbL. Using *E. coli* cell volume of 0.7 µm<sup>3</sup>, a cell division time of 20 minutes, average mRNA and protein degrade rate per hour, the energetic maintenance cost of pJP5603, transcriptional and translational expression of *wbbL* sums to roughly 9.7x10<sup>6</sup> ATP per cell (16, 17). Subtracting maintenance cost from 3.16x10<sup>9</sup> ATP per cell DFB1655 L9 possess, DFB1655 L9 still has extra 3.15x10<sup>9</sup> ATP per cells to be used. *E. coli* have roughly 2x10<sup>6</sup> copies of LPS per cell (18). If all LPS will be glycosylated with O antigen, each LPS will have 1.58x10<sup>3</sup> ATP to be used. There is no information so far about energetic cost of O antigen synthesis, but it is hard to imagine DFB1655 L9 inputting a hefty 1575 ATP per O antigen synthesis. There should be some additional processes unaccounted for. MG1655 should be complemented with *wbbL* on plasmid to ensure the sufficiency of *wbbL* in T4 resistance. An alternative would be to select for a double crossover event with clean replacement of *wbbL* in MG1655.

**Future Directions** Western blotting on WbbL and silver staining on O antigen should be performed to confirm successful antisense silencing of *wbbL*. Complementation experiment with *wbbL* on plasmid should be performed on MG1655 to confirm the sufficiency of *wbbL* in T4 resistance without introducing additional factors in genome. Sequencing of pHN678 vector given reveals a 38 bp insert within it. Therefore, pHN678 without any insert sequence should be acquired for a true empty vector control for asRNA induction.

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