The Journal of Experimental Microbiology & Immunology+

Vol 3:70-79

O16 Serotype O Antigen Expression in *Escherichia coli K-12* May Confer Resistance Against T4 Bacteriophage Infection by Preventing Adsorption

Amir Wachtel, Alice Guo, Zach Sagorin, and Emmanuel Etti
Department of Microbiology and Immunology, University of British Columbia

T4 bacteriophage infection of *Escherichia coli* strain K-12 has been well established as a model for studying virus–host interactions. Lipopolysaccharide (LPS) structure has been shown to be key for such interactions and can impact T4 bacteriophage adsorption and infection. Recently, Chiu *et al.* demonstrated that *E. coli* K-12 substrain DFB1655 L9, which expresses O antigen, is resistant to T4 infection whereas the isogenic strain MG1655, which lacks O antigen, is susceptible. The mechanism of DFB1655 L9 resistance to T4 infection is unknown. We hypothesized that O antigen expressed on the surface of *E. coli* K-12 substrain DFB1655 L9 would decrease T4 bacteriophage adsorption. Since O antigen is located on the outermost portion of LPS we assumed that it sterically blocks the T4 receptors on the core region of LPS. To test this hypothesis, we evaluated differential phage adsorption between strains MG1655 and DFB1655 L9. We used a qPCR assay to measure T4 DNA concentration in *E. coli* culture supernatants that had been incubated with T4 bacteriophage. Supernatants of *E. coli* K-12 substrain MG1655 incubated with T4 showed up to 4.9 x 10^6 fewer copies of the T4 *gp23* gene as compared to supernatants of substrain DFB1655 L9. Our data provide preliminary evidence suggesting that O antigen prevents T4 adsorption and confers resistance to infection of *E. coli* strain DFB1655 L9.

The cell envelope of Gram negative bacteria consists of an inner cellular membrane, a thin peptidoglycan layer, and an outer membrane (1). In *Escherichia coli*, the outer membrane serves as a protective barrier against foreign substances as well as the site of adsorption for various bacteriophages (2-4). The outer membrane is organized asymmetrically with a phospholipid inner layer and a lipopolysaccharide (LPS) outer layer. LPS is made up of lipid A covalently bound to a large polysaccharide core that may then be bound to an O antigen exposed on the outermost surface of the cell (3). O antigen is made up of sets of repeating glycan polymers which usually have 2-8 residues from various sugars (5, 6). The types of sugars present, the arrangement of the sugars within the polymers, and the linkages within and between the polymers can all vary resulting in approximately 170 known unique O antigen structures (6, 7). LPS is described as 'smooth' if the O antigen is present and 'rough' if the O antigen is absent (3). The outer membrane also contains a variety of proteins that regulate substances entering and exiting the cell, such as outer membrane protein C (OmpC) (2, 4, 8). Outer membrane proteins, the LPS core region, and O antigens attached to LPS serve as binding sites for some bacteriophage (3, 6). Alteration of external outer membrane structures can affect cellular resistance to bacteriophage infection by limiting access to, or directly altering, adsorption sites (8). Some O antigens serve as attachment points for bacteriophages. In other cases O antigen has shown protective effects, particularly as a defence against T4 phage infection (9, 10). Most *E. coli* K-12 strains, including substrain MG1655, lack O antigen and are described as 'rough' (10).

T4 bacteriophage is a double-stranded DNA virus that consists of a hemi-icosahedral head, a cylindrical tail, and short and long tail fibers, which together form mature virus particles (11). Consisting of 40 structural proteins and containing 274 open reading frames, T4 bacteriophage has a diverse range of hosts, including *E. coli* K-12 (11). Long and short tail fibers are necessary for T4 attachment to *E. coli*. When infecting *E. coli* K-12, the distal end of the T4 long tail fiber reversibly binds to glucose residues of LPS and OmpC on the bacterial outer membrane to initiate adsorption (11, 12). Upon long tail fiber engagement, T4

Received: 09/06 2017 **Accepted:** 10/17 2017 **Published:** 11/15 2017

short tail fibers extend from the baseplate and irreversibly bind to a heptose residue on the LPS core structure (12). Following irreversible binding, the T4 tail tube punctures the *E. coli* cell envelope with the help of a lysozyme protein at the end of the tail tube (13). The viral genome is then injected into *E. coli*, where viral replication occurs prior to cell lysis (13). LPS is essential for phage infectivity and propagation (4). A key T4 bacteriophage gene used in this study is *gp23*. *gp23* encodes a T4 major capsid protein and was used to identify and enumerate T4 bacteriophage (9).

Chiu *et al.* recently showed that strain DFB1655 L9 is resistant to T4 infection whereas isogenic strain MG1655 is not (9). MG1655 carries an insertion mutation of a 1,195 base pair IS5 element in the *wbbL* gene of the *rfb* cluster (14). The *wbbL* gene encodes the protein rhamnose transferase, which allows for biosynthesis of O antigen serotype O16 (10, 14). The IS5 insertion element prevents O antigen synthesis in MG1655 (9, 10). The *wbbL* gene is reintroduced into MG1655 by a single cross over chromosomal insertion of an intact version of the gene, resulting in the newly modified substrain DFB1655 L9 (10). *E. coli* K-12 substrain DFB1655 L9 synthesizes O antigen serotype O16 and has been shown to be highly resistant to T4 bacteriophage infection (9). Other factors influencing phage infectivity, such as growth rate and outer membrane composition, are similar between DFB1655 L9 and MG1655 substrains (10). It was concluded that O antigen synthesis confers resistance to T4 infection in *E. coli* strain DFB1655 L9, however, the mechanism by which *E. coli* K-12 substrain DFB1655 L9 resists infection remains unknown (9).

In this study, we aimed to test whether O antigen synthesis results in decreased adsorption of T4 bacteriophage to *E. coli* K-12 substrain DFB1655 L9. Since O antigen is displayed on the outermost portion of the *E. coli* outer membrane, we hypothesized that synthesis of O antigen serotype O16 in *E. coli* K-12 substrain DFB1655 L9 would decrease T4 adsorption, potentially by preventing access to glucose and heptose residue adsorption sites in the LPS layer (3). Preventing T4 bacteriophage adsorption to the outer membrane surface of *E. coli* could prevent infection. To test our hypothesis, we used an adsorption assay and measured the amount of T4 gp23 genes found in culture supernatants that had been incubated with T4 using qPCR. The number of gp23 is representative of the number of unbound T4 phage particles present in each supernatant. Our data suggest that O antigen inhibits adsorption of T4 to *E. coli* K-12 substrain DFB1655 L9.

MATERIALS AND METHODS

Strains used in this study. *E. coli* K-12 substrains MG1655 and DFB1655 L9 were a gift from Dr. Douglas F. Browning from the Henderson laboratory at the University of Birmingham (10). MG1655 is a commonly used laboratory strain with an IS5 insertion within the *wbbL* gene of the *rfb* locus, which prevents O antigen production (10). The DFB1655 L9 substrain was generated from MG1655 following the complementation and insertion of the *wbbL* gene into the chromosome of MG1655, which restored O16 serotype O antigen synthesis (10). Bacteriophage T4 was initially obtained from the Carolina Biological Supply (cat no. 12-4330) and was available from a previous study done in our laboratory (9).

Isolation of colonies of *E. coli* K-12 substrain MG1655 and DFB1655 L9. The MG1655 substrain was streaked onto 1.5% agar Lysogeny Broth (LB) agar plates and the DFB1655 L9 substrain was streaked on LB agar plates supplemented with 50 µg/mL kanamycin salt (Invitrogen). Both plates were incubated overnight at 37°C. Isolated colonies from both plates were then streaked on fresh media and incubated overnight at 37°C.

PCR strain identity confirmation. Overnight cultures of MG1655 and DFB1655 L9 in 5 mL of LB were generated and genomic DNA of each substrain was isolated using the PureLink™ Genomic DNA Mini Kit (Catalog No K1820-00) according to the included kit protocol. NanoDrop 2000c spectrophotometer by Thermo Scientific was used to assess DNA purity and concentration. PCR amplification was performed on the isolated genomic DNA of both strains using the Platinum® Taq DNA Polymerase (Invitrogen) kit, according to the manufacturer's instructions. Primers specific to the *wbbL* gene were sourced from Browning *et al*. and were available in the laboratory from previous use by Chiu *et al*. (9, 10). Primers were used at final concentrations of 0.2 µM per reaction sample. Template DNA was used at a final amount of 155 ng per reaction sample. The Bio-Rad T100™ Thermal Cycler was set to a 5 minute initial denaturation at 95°C and then 30 cycles consisting of: a 95°C denaturation phase for 30 seconds, a 55°C annealing phase for 45 seconds, and a 75°C extension phase for 2.5 minutes. PCR products were run on a 0.8% agarose gel using 1x TAE buffer at 80V for 100 minutes and visualized using SYBR™ Safe DNA Gel Stain (Invitrogen) under an ultraviolet (UV) scan.

Phage lysate generation. Based on methods designed by Chiu *et al.*, *E. coli* MG1655 was inoculated in 5 mL of LB and grown overnight at 37°C (9). The overnight culture of MG1655 was diluted by 1/5 in 5 mL of LB. The culture dilution was inoculated with 10uL of T4 bacteriophage obtained from Chiu *et al.* and grown overnight at 37°C on a shaking platform (200 rpm) to generate the T4 lysate (9). 300 µL of chloroform was added to the lysate the following day, it was then vortexed, and left overnight at 4°C to settle. The 1/5 culture dilution resolved to be nearly translucent and was used for T4 phage extraction. Chloroform extraction was performed using sterile filtration on the T4 lysate to obtain a working T4 bacteriophage stock. A T7 lysate was obtained from Francis *et al.* and was used as a negative control for the phage identity confirmation (Figure 2). Later in the study, LB media that was prepared for the T4 bacteriophage was supplemented with 1mM $CaCl_2$, as this increased plaque formation.

Phage identity confirmation using PCR. PCR amplification of the T4 lysate was evaluated using T4-specific *gp23* gene primers and T7-specific *gp10a* gene primers using the Platinum Taq DNA polymerase kit (Invitrogen) as per the manufacturer's instructions (Table 2). *gp23* encodes a T4 major capsid protein, while *gp10a* encodes a T7 major capsid head protein and both have been used to identify their respective viruses in past studies (9). The T7

primers were used as a negative control to ensure the purity of the T4 lysate. The primers were ordered from Integrated DNA Technologies and used at final concentrations of 0.2 µM. 1µL of phage lysate was added to each reaction sample. The Bio-Rad T100 Thermal Cycler was programmed to include a 2-minute initial denaturation step at 95°C followed by 30 cycles including: denaturation for 45 seconds at 95°C, annealing for 30 seconds at 51°C, and extension for 30 seconds at 74°C. All PCR products were run on a 1.2% agarose gel in 1X TAE buffer at 110V for 55 minutes. Bands were visualized using 10 µL of Invitrogen's SYBR™ Safe DNA Gel Stain.

Table 1. *E. coli* K-12 strain MG1655 *wbbL*-specific gene primers. F and R represent forward and reverse primers, respectively.

Gene	Sequence (5′ - 3′)	Size (bp)
wbbL	F: CCCGAATTCATATGGTAT ATATAATAATCGTTTCCC	1994 (MG1655)
	R: CCCAAGCTTCTCGAGTTACG GGTGAAAAACTGATGAAATTC	799 (DFB1655 L9)

Table 2. Bacteriophage primers designed to amplify T4 capsid protein gene *gp23* and T7 gp23a. The letter F denotes the forward primer and the letter R denotes the reverse primer.

Gene	Sequence (5′ - 3′)	Size (bp)
T4 *gp23*	F: GCCATTACTGGAAGGTGAAGG R: TTGGGTGGAATGCTTCTTTAG	398
T7 *gp10a*	F: CGAGGGCTTAGGTACTGC R: GGTGAGGTGCGGAACTTC	295

Double agar overlay plaque assay to enumerate T4 phage titre and confirm differential susceptibility of *E. coli* K-12 MG1655 and DFB1655 L9 to T4 infection. All reagents and methods used to perform the double agar overlay plaque assay were derived from Chiu *et al*. and Kropinski *et al*. (9, 15). The LB supplemented with $CaCl_2$ (LB+$CaCl_2$) was prepared for the underlay agar using an agar density of 15 g/L. The LB+$CaCl_2$ overlay agar was prepared with a final agar density of 4 g/L. Both the underlay and overlay agar solutions were supplemented with 1 mM of $CaCl_2$ because it facilitates T4 infectivity (9). Approximately 18-25 mL of the underlay agar solution was poured into 14 plastic petri dishes, cooled, and stored at 4°C. The overlay agar was distributed into 3mL aliquots and stored overnight at 4°C. Prior to use, all glass tubes carrying overlay agar broths were passed over a bunsen burner to melt the solidified overlay agar solution and then placed in a 56.8°C water bath to prevent it from solidifying again. All underlay agar plates were placed in a 37°C incubator for approximately 105 minutes to dry any remaining condensation prior to plating. A serial dilution of the previously purified T4 lysate (10^{-1} to 10^{-9}) was performed in LB. OD_{600} spectrophotometer readings were completed on both MG1655 and DFB1655 L9 *E. coli* K-12 substrains. 100µL of the 10^{-4} to 10^{-9} T4 dilutions and 100µL of either MG1655 or DFB1655 L9 overnight cultures were added to the liquid overlay agar. The solution was mixed and immediately plated on the LB+$CaCl_2$ plates. All plates were incubated overnight at 37°C. The number of plaque forming units (PFUs) was counted the following day. The 10^{-6} dilution of T4 presented a PFU within the 30-300 countable range and was used to determine the titre of the T4 bacteriophage stock solution, which was calculated to be 1.7 x 10^9 PFU/ml.

Preparing *E. coli* cells for adsorption assay. LB broth was used to prepare 50 mL overnight cultures of *E. coli* K-12 substrains DFB1655 L9 and MG1655. The overnight cultures were then diluted to an optical density (OD) of 1, as measured using a Pharmacia Biotech Ultrospec 3000 at 600 nm. The OD_{600} of the normalized samples was then used to determine the volume of each sample required to obtain 1.7x10^9 cells. The calculated volume was then pipetted into 2.0 ml centrifuge tubes and centrifuged at 16,000 rcf for 3 minutes. The supernatant was discarded, and the pellets were resuspended in 900 µL of LB.

Preparing bacteriophage T4 dilutions for adsorption assay. The plaque assay revealed that our phage lysate concentration was 1.7 x 10^9 PFU/mL. 50µL of this lysate was added to 4.95 mL of LB to create a 1/100 working stock of 1.7 x 10^4 PFU/µL. The working stock was then serially diluted, with 500 µL of the previous solution being transferred to 4.5 mL of LB to create solutions of 1.7 x 10^4, 1.7x10^3, 1.7x10^2, 17, 1.7 and 0.17 PFU/µL.

T4 adsorption assay. T4 phage were incubated with each *E. coli* strain at equal volumes and decreasing multiplicities of infection (MOIs) from 10^{-3} to 10^{-8}. Each MOI was calculated by dividing the number of PFU in each sample by the number of *E. coli* cells in each sample. Therefore, at an MOI of 10^{-3}, there were 1.7 x 10^6 phage particles and 1.7 x 10^9 *E. coli* cells in the test tube. Each assay was performed with two replicates using a staggered start timing method to ensure equivalent incubation times. Working from the lowest phage concentration to the highest, 100 µL of the diluted phage was added at 0 minutes to a prepared 900 µL *E. coli* K-12 cell suspension. The next phage dilution was introduced to a new cell suspension every 4 minutes. The cells were allowed to incubate with the virus on the bench top for 5 minutes and then immediately centrifuged for 3 minutes at 16,000 x g. T4 and *E. coli* were incubated for 5 minutes so as to only allow adsorption to occur as T4 bacteriophage requires approximately 25 minutes to induce cell lysis (16). Upon completing the centrifugation, 800 µL of the supernatant was immediately transferred to a new sterile centrifuge tube. 100 µL of chloroform was then added to the supernatant to disrupt any remaining cells and prevent viral replication. The chloroform and supernatant mixtures were shaken by hand and allowed to rest on the bench or stored in a 4°C fridge. The same protocol was followed for the controls except that LB was added to the *E. coli* cells in lieu of T4 bacteriophage for the negative controls, and 900 µL of LB + 100 µL of the various phage dilutions, without the presence of *E. coli* cells, were used as positive controls. The positive controls were run in duplicates and will be referred to as our Standard samples.

qPCR for adsorption assay and standard curve generation. The primers were designed to amplify a 150bp section of the T4 *gp23* gene as shown in Table S2. Primers were ordered from Integrated DNA Technologies (IDT) and used at final concentrations of 0.1µM. A master mix containing the required water and forward and reverse primers for all reactions was then prepared. In a biosafety cabinet the following reagents were combined into each qPCR well of a 96-well plate: 12 µL of the master mix, 0.5 µL of the test supernatant, and 12.5 µL SYBR® Green Master Mix. The 96-well plate was then transferred to a Bio-Rad T100™ Thermal Cycler. The Thermal Cycler was programmed for a two-step amplification followed by melt curve analysis. The amplification was programmed for a 10 minute, 95°C initial denaturation step, followed by 39 cycles made up of a 95°C denaturation step for 15 seconds and a 30 second annealing

step at 50°C. The data was then collected and analyzed using the Bio-Rad CFX Manager™ Software and Microsoft® Excel® 2011. The Cq values for the Standard samples were plotted against the number of phage (Figure 4A). This graph was used as a standard curve and its slope was used to convert the Cq values of the experimental samples into the number of phage in each sample.

RESULTS

PCR analysis of *E. coli* K-12 DFB1655 L9 genomic DNA using primers flanking *wbbL* yields a band of 800 base pairs consistent with the expected size of the *wbbL* gene. To characterize strain DFB1655 L9 we used primers flanking the 799 base pair (bp) *wbbL* gene for PCR (9). As expected, PCR amplification of strain MG1655 resulted in a product corresponding to the 2 kbp non-functional *wbbL* gene as seen in Figure 1 (9, 10). The DFB1655 L9 PCR resulted in a 2 kbp, a 1.5 kbp, and an 800 bp product (Figure 1). The 800 bp band corresponds to the expected size of the functional *wbbL* gene in DFB1655 L9 (9, 10). The approximately 2.0 kbp band in the DFB1655 L9 is present due to the *wbbL* gene being introduced via a single crossover, which resulted in the presence of both functional and non-functional *wbbL* genes in the DFB1655 L9 chromosome (10). Our gel electrophoresis results are consistent with previous findings by Chiu *et al* (9). The additional band near the 1.5 kbp amplicon present in the DFB1655 L9 PCR product suggests a possible non-specific binding site for the primers. Additionally, a smear was present at the bottom of the negative control lane, indicating potential primer interactions in the absence of the binding sequence of interest (Figure 1). Taken together these data show the expected *wbbL* genotypes for strains DFB1655 L9 and MG1655.

PCR analysis of bacteriophage lysate shows amplification of T4 *gp23* but not T7 *gp10*. Since our laboratory studies both T4 and T7 bacteriophage, we used PCR to confirm the identity of our stock bacteriophage lysate using primer pairs specific to genes *gp23* and *gp10a,* which are unique to bacteriophages T4 and T7, respectively. The PCR resulted in a product consistent with the expected size of *gp23* in T4 bacteriophage (Figure 2). Our positive control T4 lysate resulted in a PCR product of approximately 400 bp, shown in Figure 2. PCR using T7 DNA and T7 specific primers yielded a band at the anticipated length of approximately 300 bp. The absence of a similar band following PCR of the stock lysate with T7 specific primers indicated that there was no T7 contamination in the generated lysate (Figure 2, Lane T7 P + T4 Lys). Negative controls using distilled water and only T7 or T4 specific primers resulted in no product demonstrating that the primers used do not interact with one another to produce primer-dimers or other nonspecific PCR products in the absence of the target sequence of interest. Collectively, this PCR assay confirmed that the phage stock generated was T4 bacteriophage that was not contaminated with T7 bacteriophage.

***E. coli* K-12 substrain DFB1655 L9 is resistant to T4 infection.** Chiu *et al*. showed that substrain DFB1655 L9 exhibits greater resistance to T4 bacteriophage than

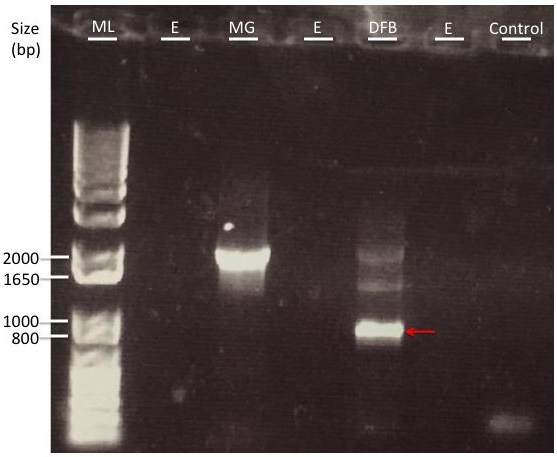

Figure 1. *Escherichia coli* K-12 strain DFB1655 L9 possesses the *wbbL* gene necessary for O16 antigen production, whereas MG1655 does not. PCR was performed on genomic DNA isolated from the two strains. The intact *wbbL* gene is only present in the DFB1655 L9 strain and is at ~800bp (red square). The ~2kbp amplicon is present in both strains and indicates the non-functional *wbbL* gene with the 1.2kbp IS5 insertion mutation. The lane labelled Control used *wbbL*-specific primers and H_2O in place of DNA template. ML = Molecular Ladder; E = Empty; MG = MG1655 DNA; DFB = DFB1655 L9 DNA.

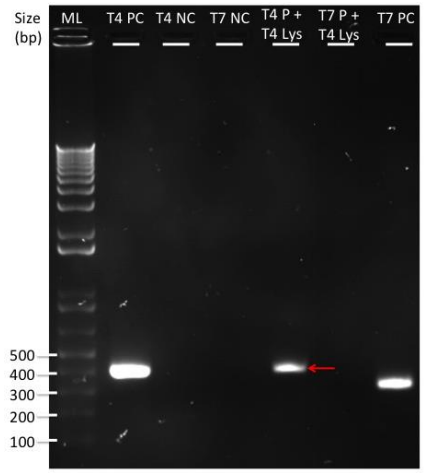

Figure 2. Confirmation that T4 lysate is free of T7. A single band at ~398bp in the T4P + T4Lys test lane corresponds with the T4 positive control, does not correspond with the T7 positive control, and indicates that a pure T4 lysate sample without T7 contamination was generated. T4 primers amplified the T4 *gp23* gene and T7 primers amplified the T7 *gp10a* gene. These were used to propagate phage genes in a lysate sample generated from an overnight bacterial culture. ML = Molecular Ladder; P = Primers; Lys = Lysate, NC = Negative Control; PC = Positive Control.

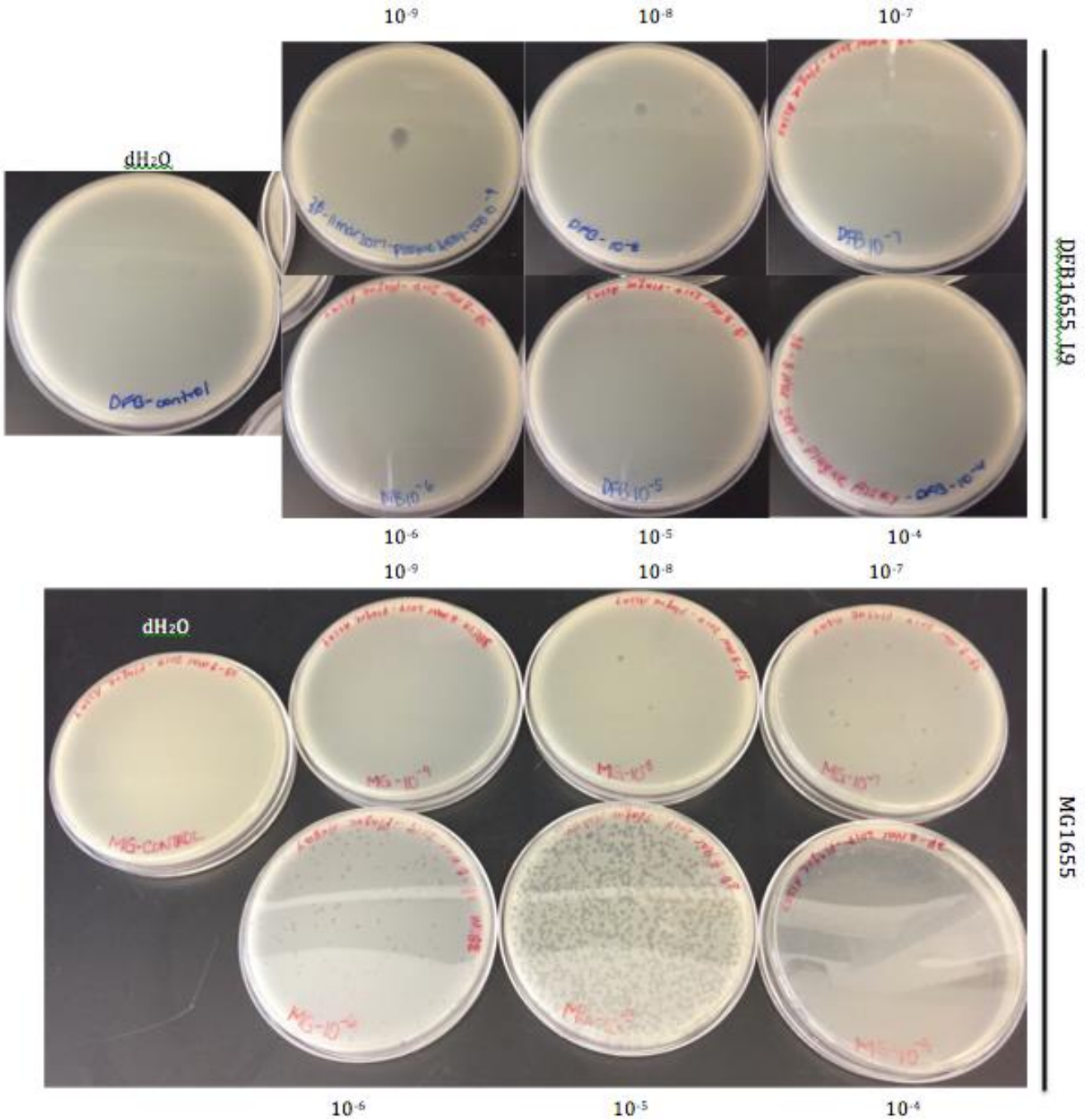

Figure 3. DFB1655 L9 shows resistance to bacteriophage T4 infection-mediated lysis. DFB1655L9 shows no plaques at all phage titres tested. MG1655 forms plaques at all phage titres tested except the 10^{-9} dilution. The control plates contain their respective *E. coli* strain and distilled water substituted for phage. LB plates containing $CaCl_2$ were incubated at 37°C.

MG1655 due to differential O16 antigen expression (9). To confirm MG1655 sensitivity and DFB1655 L9 resistance to T4 phage infection we performed a double agar overlay plaque assay in which serial dilutions of T4 phage were plated with either *E. coli* K-12 substrains MG1655 or DFB1655 L9 and incubated overnight. OD_{600} readings of the MG1655 and DFB1655 L9 overnight cultures measured 1.8 and 1.5, respectively. Plaques were counted the next day (Figure 3). All T4 containing MG1655 substrain plates showed plaques, with the exception of the 10^{-9} dilution of T4 phage, where no plaques were observed (Figure 3). The 10^{-6} dilution of T4 phage generated 170 plaques, which was used to enumerate the viral load in the purified T4 lysate solution, yielding a value of 1.70×10^9 PFU/mL. None of the DFB1655 L9 plates showed plaques and they all grew into confluent bacterial lawns following overnight

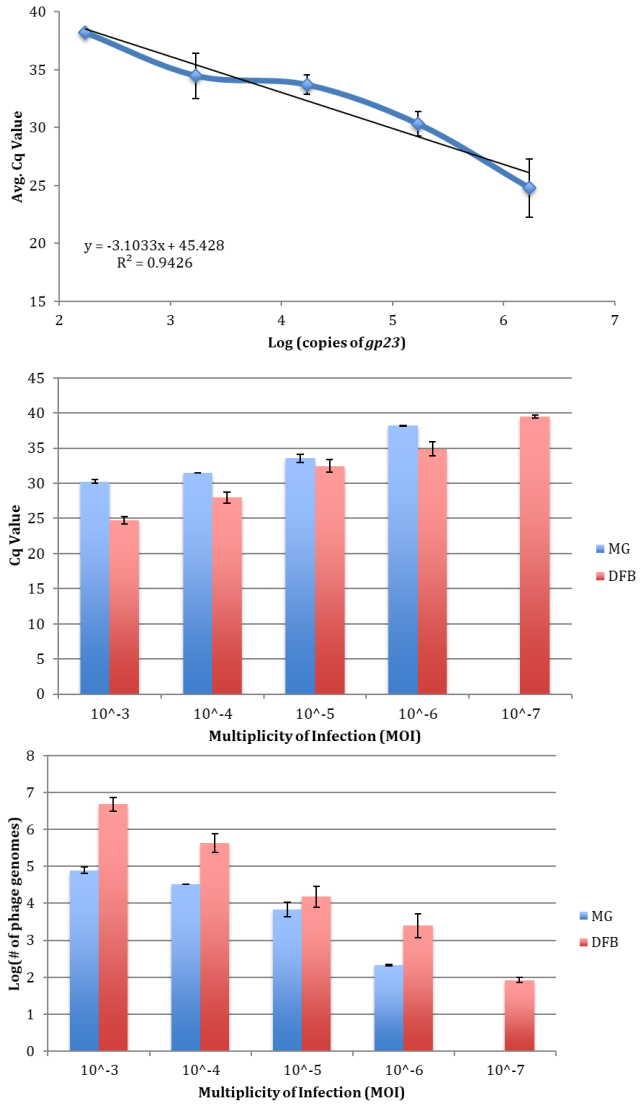

Figure 4. (A) Standard curve of T4 bacteriophage qPCR. 1.7 x 10^4 PFU/µL in LB was serially diluted to create a total of 6 solutions decreasing PFU/µL. In duplicates, 100 µL of each dilution was combined with 900 µL of LB. The number of T4 bacteriophage in each replicate was quantified using qPCR. The primers are described in Table S2 and the amplification efficiency of the reaction was reported to be 110.1% (Thermo Fisher Scientific qPCR efficiency calculator). Error bars report standard deviation. **(B)** Cq values of supernatants of T4 bacteriophage-exposed *E. coli* K-12 substrains MG1655 and DFB1655 L9. Experiments were performed at multiplicities of infection ranging from 10^{-3} to 10^{-7}. The data was generated by measuring two replicates per MOI and calculating their average. MG = MG1655 and DFB = DFB1655 L9. Error bars report standard deviation. **(C)** Enumeration of T4 bacteriophage remaining in culture supernatants following adsorption. T4 were quantified using qPCR and previously designed primers (Table S2). The T4 bacteriophage standard curve was used to convert Cq values into Log[number of phage] at each MOI (Figures 4A and 4B). The data was generated by measuring two replicates per MOI and calculating their average. Error bars report standard deviation. MG = MG1655 supernatant and DFB = DFB1655 L9 Supernatant.

incubation (Figure 3). As controls, 100µL of dH_2O was added to 100µL of both MG1655 and DFB1655 L9. These solutions were plated and then incubated overnight. Both controls showed no plaque formation and grew into confluent lawns (Figure 3). These results are consistent with those of Chiu *et al*. showing that strain DFB1655 L9 is resistant and that strain MG1655 is susceptible to T4 infection (9).

Supernatants of DFB1655 L9 contain more T4 bacteriophage than supernatants of MG1655. In order to compare adsorption of T4 bacteriophage in *E. coli* K-12 substrains MG1655 and DFB1655 L9, an adsorption assay was used where T4 phage particle concentration was measured using qPCR. The adsorption assay involved incubating suspensions of MG1655 or DFB1655 L9 cells with varying viral concentrations of T4 to achieve multiplicities of infection (MOIs) of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}. The negative controls, containing *E. coli* cells only, exhibited no qPCR signal (data not shown). Virus particles in the absence of *E. coli*, were used as positive controls to build a standard curve to correlate PFU with each Cq measurement (Figure 4A). The number of T4 added to each positive control is the same number of T4 added to each experimental MOI sample. Therefore, because the positive controls have no *E. coli* cells present, they represent the maximum amount of virus present in the supernatant at each MOI tested. A melt curve analysis generated a single peak at 85°C indicating that only one gene product was amplified in all the samples with detectable levels of phage (Figure S1). Greater numbers of the T4 *gp23* gene were recovered from the DFB1655 L9 supernatants than MG1655 supernatants at all the MOIs tested (Figure 4C). This was most evident at the 10^{-3} MOI where an average of $4.9x10^6$ more copies of the T4 *gp23* gene were detected in the DFB1655 L9 supernatants than MG1655 supernatants (Figure 4C). Furthermore, the MG1655 supernatant samples showed no pPCR signal at MOIs of $1x10^{-8}$ and $1x10^{-7}$ whereas the DFB1655 L9 supernatant samples showed no signal at an MOI of $1x10^{-8}$ and showed a high average Cq value of 39.5 at an MOI of $1x10^{-7}$ (Figure 4B). The rationale for using low MOIs was to potentially identify a point at which all the phage in solution was absorbed by one strain but not the other. This was observed at the MOI of 10^{-7}, at which no signal was detected for the MG1655 replicates while a signal was detected for the DFB1655 replicates (Figure 4C). No statistical analysis was performed due to the low sample size of two replicates.

DISCUSSION

Previous studies have shown that expression of O antigen serotype O16 in *E. coli* K-12 confers resistance against T4 bacteriophage infection (9). The mechanism for this resistance is unknown. In this study, we aimed

to test whether O antigen synthesis results in decreased bacteriophage adsorption to determine if decreased adsorption was a viable explanation for the aforementioned resistance. We hypothesized that the presence of O antigen serotype O16 oligosaccharide on *E. coli* strain DFB1655 L9 LPS inhibits T4 adsorption. We first confirmed the identity of the *E. coli* substrains using PCR (Figure 1). We then generated a phage lysate and confirmed the phage identity through another PCR analysis (Figure 2). Next, we performed a double-overlay plaque assay to confirm that strain DFB1655 L9 is resistant to T4 infection whereas MG1655 is not (Figure 3). Finally, we tested whether O antigen expression results in decreased levels of T4 bacteriophage adsorption (Figure 4). To do this we used an assay that quantified unabsorbed T4 bacteriophage in culture supernatants using qPCR.

The assay evaluates differential adsorption by comparing the amount of T4 bacteriophage that failed to adsorb to either MG1655 or DFB1655 L9 cells following short-term incubation. T4 that do not bind to *E. coli* should be present in the supernatant and can be detected using qPCR. If the two *E. coli* strains show differential adsorption of T4 bacteriophage there should be a difference in the signal detected via a qPCR of their respective supernatants. Both *E. coli* strains are known to have equivalent growth rates, outer membrane compositions, and share other characteristics that influence phage adsorption kinetics. The only notable difference between them is that DFB1655 L9 has O antigen on its most exterior portion while MG1655 does not (10). Therefore, we reasoned that a difference in the adsorption between these two *E. coli* K-12 substrains would likely be due to the expression of O antigen.

For this assay, we chose to quantify the number of phage particles present in the supernatants via qPCR, as opposed to performing plaque assays. Plaque assays are often considered the "gold standard" for phage enumeration because they only quantify infective phage particles (17). However, while qPCR analysis may quantify non-infective phage particles it has the advantage of being less technically burdensome and can be less variable than plaque assays (17, 18). Enumerating phage by qPCR also allows for faster turnaround because only 3-4 hours are needed to obtain results rather than the 18-24 hours required when using plaque assays and it has a higher throughput capability when using a multi-well plate (17).

The data from the adsorption assay supports our hypothesis that O antigen expression inhibits T4 adsorption since the amount of T4 bacteriophage present in the supernatant of DFB1655 L9 is higher across every MOI than that of MG1655 (Figure 4C). This data suggests that less bacteriophage adsorbed to DFB1655 L9 than MG1655. At the MOIs of 10^{-3},10^{-4}, and 10^{-6} a 10-fold difference in the number of T4 *gp23* genes was observed between the DFB1655 L9 and MG1655 supernatants (Figure 4C). This difference in bacteriophage adsorption between the two strains is potentially further supported by the finding that T4 bacteriophage was absent from MG1655 supernatants beginning one serial dilution earlier than DFB1655 L9 (Figure 4C). On the one hand, the Cq value of 39.5 detected at the 10^{-7} MOI for the DFB1655 L9 supernatant is very high and should be treated with caution. On the other hand, no signal was detected for the negative controls in this experiment. Further testing is required to elucidate the limit of detection for the qPCR in the adsorbance assay.

No statistical analysis was performed due to the small sample size of two replicates per MOI tested. A two-sample t-test requires that the data be normally distributed or, if there are minor deviations from normality, that the sample size be greater than or equal to 5 (19). Because of the small sample size, we cannot test the distribution of the data nor can we calculate the degree to which it potentially deviates from normality.

According to the adsorption assay there was a greater amount of T4 DNA in the DFB1655 L9 supernatants than the standards at MOIs of 10^{-3}, 10^{-4}, and 10^{-5} (Figure S2). This finding implies that there was a greater than 100% recovery in these samples as the standards represent the maximum amount of T4 that could be recovered at each MOI. Three potential explanations for this are that (i) the presence of the *E. coli* cells in the experimental samples may be altering environmental conditions which is in turn altering the qPCR efficiency, (ii) that the phage are replicating and lysing the *E. coli* cells prior to chloroform treatment, and (iii) that variability in our assay is responsible for this observation. These scenarios are discussed below.

While the *E. coli* cells in the experimental samples could alter the environmental conditions to increase primer / template hybridization rates only $1/50^{th}$, 0.5 µL out of 25 µL, of each qPCR solution would have come from the different samples. Therefore, even if the cells altered the composition of the DFB1655 L9 supernatant compared to the standards, because the volume in the qPCR tubes resulting from this supernatant is so small, this explanation is likely not responsible for the noted finding.

T4 bacteriophage replicating and lysing the DFB1655 L9 cells is an unlikely explanation for the greater than 100% phage recovery. Firstly, T4 bacteriophage requires approximately 25 minutes to induce cell lysis, while we only incubated the *E. coli* cells with T4 for 5 minutes (16). Secondly, in Figure 2 we showed that DFB1655 L9 is completely resistant to T4-mediated cell lysis, which would prevent an increase in viral titre in the supernatants of the DFB1655 L9 cells due to infection.

Finally, the differences in the number of T4 recovered from the DFB1655 L9 supernatants and the standards could be due to variability in our study. Because our sample sizes are small (n=2) we cannot statistically assess this variability. If the differences were due to chance it would mean that the amount of phage recovered from the DFB1655 L9 represents the maximum amount we could recover from each supernatant and is essentially equal to the standards. That would mean that O antigen expression is able to completely block T4 adsorption, which would explain why DFB1655 L9 is completely resistant to T4 bacteriophage infection (Figure 2). That being said, for all the MOIs tested the DFB1655 L9 strain had more phage than MG1655. This consistency argues against assay variability and, therefore, this observation should be further investigated to determine its cause.

The mechanism responsible for the T4 resistance of DFB1655 L9 is unknown. Some potential explanations include: (i) O antigen could bind T4 thereby initiating attachment of the bacteriophage to its host at the incorrect receptor, (ii) it could bind T4 and cause the bacteriophage to prematurely release its genome, (iii) the DFB1655 L9 cells could shed O antigen which would bind the bacteriophage so that it could not bind the actual cell, or (iv) it could sterically prevent access to the LPS core receptor preventing any attachment to the host cell.

Our data indicates that O antigen results in decreased adsorption. That would mean that O antigen expression likely does not block access to key T4 LPS receptors by binding the bacteriophage at another incorrect receptor. If that were the case, both MG1655 and DFB1655 L9 would be able to pull T4 out of solution and we would expect equal numbers of bacteriophage to be present in the MG1655 and DFB1655 L9 supernatants, which was not observed (Figure 4C).

It is worth noting that the initial denaturation step of all our qPCR analyses resulted in the breakdown of the viral capsid and release of the viral genome from all bacteriophage present. Therefore, our assay did not discriminate between decreased adsorption and premature viral genome release.

Chiu *et al.* incubated T4 in the supernatant of MG1655 and DFB1655 L9 overnight cultures and found that these bacteriophages were as capable of causing infection as pure T4 bacteriophage (9). This suggests that O antigen being shed and neutralizing T4 bacteriophage is not a likely explanation for resistance to T4 infection (9).

Finally, in the mechanism of sterically preventing T4 from adsorbing to the cells, the O antigen functions as a physical barrier between the phage receptor and the bacteriophage. For example, the *E. coli* K1 capsule has been shown to directly interfere with T7 attaching to its host receptor (20). This would result in a greater number of phage being present in the DFB1655 L9 supernatant, which fits with the data shown in Figure 4C. The position of the O antigen on the most exterior portion of the *E. coli* K-12 outer membrane places it between the bacteriophage and its LPS receptor, rendering the possibility of steric hindrance a tempting mechanism to explain decreased T4 adsorption and increased resistance (21).

The results of the adsorbance assay suggest that decreased adsorption is occurring in the DFB1655 L9 samples. However, it should be noted that the assay was performed only once in this study with duplicate samples, so strong conclusions should be tempered until the experiment is repeated with a greater number of replicates and a subsequent statistical analysis is performed. Furthermore, future experiments are needed to investigate the potential of premature viral genome release as a mechanism for the observed T4 resistance of DFB1655 L9. Future researchers could consider repeating this absorption assay with higher MOIs to investigate the potential differential adsorption at higher phage concentrations.

In conclusion, we utilized a qPCR-based adsorption assay to measure T4 bacteriophage adsorption to *E. coli* K-12 substrains MG1655 and DFB1655 L9 using the supernatants of cells grown in the presence of bacteriophage T4. Consistent with our hypothesis that O antigen inhibits binding of T4 to the LPS of DFB1655 L9, we observed an increase in the phage remaining in the culture supernatant after incubation with DFB1655 L9 as compared to MG1655 at all the MOIs tested. This difference in bacteriophage adsorption between the two *E. coli* K-12 substrains was greater than 10-fold at multiple MOIs tested. Taken together these results suggest that O antigen serotype O16 expression in *E. coli* inhibits T4 adsorption.

FUTURE DIRECTIONS

To further investigate the mechanism of decreased adsorption the phage binding sites in MG1655 and DFB1655 L9 could be immunolabelled and the strength of the signal could be compared (22). We would expect the data from such an experiment to show a stronger signal for the MG1655 cells than the DFB1655 cells if the O antigen sterically protects the binding sites to the point that the antibodies cannot bind them. However, contrary data would not rule out the decreased adsorption mechanism of steric hindrance, as this mechanism could function for particles the size of a virus while not functioning for particles the size of an immunolabeling antibody. Also, the effect of O16 antigen expression on virus-mediated cell lysis and viral adsorption could be tested using other (e.g. non T4) bacteriophages. For example, T7 bacteriophage binds LPS and therefore it is possible that O16 antigen would disrupt its ability to bind the required receptors leading to resistance to T7 infection and adsorption (23). This experiment could show whether O antigen expression leads to widespread versus targeted resistance and the results of such an experiment could shed more light on the mechanism of resistance caused by O16 antigen expression. For example, if future researchers find that O16 antigen expression confers resistance to multiple LPS-binding bacteriophages it would further support the steric hindrance model of decreased adsorption as this is a non-targeted defence mechanism.

ACKNOWLEDGEMENTS

We are grateful for the support, funding, and laboratory space provided by the Department of Microbiology & Immunology at the University of British Columbia. We are especially thankful for the tremendous guidance offered by Dr. David Oliver, Angela Lin, and Gyles Ifill. We are also grateful to our laboratory colleagues Freddy Francis, Rachel Miller, and Manny Nagra who supplied us with the T7 lysate and Yvonne Ma who helped with troubleshooting and protocol development, and the media room staff for all of the supplies they made available for our use.

REFERENCES

1. **Beveridge, TJ.** 1999. Structures of gram-negative cell walls and their derived membrane vesicles. J. Bacteriol. **181:**4725-4733.
2. **Washizaki, A, Yonesaki, T, Otsuka, Y.** 2016. Characterization of the interactions between Escherichia coli receptors, LPS and OmpC, and bacteriophage T4 long tail fibers. MicrobiologyOpen. **5:**1003-1015.
3. **Rakhuba, DV, Kolomiets, EI, Dey, ES, Novik, GI.** 2010. Bacteriophage receptors, mechanisms of phage adsorption and penetration into host cell. Polish Journal of Microbiology / Polskie Towarzystwo Mikrobiologów = the Polish Society of Microbiologists. **59:**145.
4. **Yu, F, Mizushima, S.** 1982. Roles of lipopolysaccharide and outer membrane protein OmpC of Escherichia coli K-12 in the receptor function for bacteriophage T4. J. Bacteriol. **151:**718-722.
5. **Lerouge, I, Vanderleyden, J.** 2002. O-antigen structural variation: mechanisms and possible roles in animal/plant–microbe interactions. FEMS Microbiol. Rev. **26:**17-47.
6. **Wang, L, Wang, Q, Reeves, PR.** 2010. The Variation of O Antigens in Gram-Negative Bacteria, p. 123-152. *In* Anonymous vol. 53. Springer Netherlands, Dordrecht.
7. **Raetz, CRH, Whitfield, C.** 2002. Lipopolysaccharide endotoxins. Annu. Rev. Biochem. **71:**635-700.
8. **P van der Ley, Graaff, Pd, Tommassen, J.** 1986. Shielding of Escherichia coli outer membrane proteins as receptors for bacteriophages and colicins by O-antigenic chains of lipopolysaccharide. J. Bacteriol. **168:**449-451.
9. **Chiu, J, Croft, C, Ng, K.** 2017. *Escherichia coli* O antigen seroptype O16 is a restriction factor for bacteriophage T4 infection. : 1-36. Jemi. **21:**1-36.
10. **Browning, DF, Wells, TJ, França, FLS, Morris, FC, Sevastsyanovich, YR, Bryant, JA, Johnson, MD, Lund, PA, Cunningham, AF, Hobman, JL, May, RC, Webber, MA, Henderson, IR.** 2013. Laboratory adapted Escherichia coli K-12 becomes a pathogen of Caenorhabditis elegans upon restoration of O antigen biosynthesis. Mol. Microbiol. **87:**939-950.
11. **Leiman, PG, Kanamaru, S, Mesyanzhinov, VV, Arisaka, F, Rossmann, MG.** 2003. Structure and morphogenesis of bacteriophage T4. Cellular and Molecular Life Sciences. **60:**2356-2370.
12. **Ali, SA, Iwabuchi, N, Matsui, T, Hirota, K, Kidokoro, S, Arai, M, Kuwajima, K, Schuck, P, Arisaka, F.** 2003. Reversible and Fast Association Equilibria of a Molecular Chaperone, gp57A, of Bacteriophage T4. Biophys. J. **85:**2606-2618.
13. **Kostyuchenko, VA, Kanamaru, S, Mesyanzhinov, VV, Arisaka, F, Chipman, PR, Leiman, PG, Rossmann, MG.** 2002. Structure of the cell-puncturing device of bacteriophage T4. Nature. **415:**553-557.
14. **Stevenson, G, Neal, B, Liu, D, Hobbs, M, Packer, NH, Batley, M, Redmond, JW, Lindquist, L, Reeves, P.** 1994. Structure of the O antigen of Escherichia coli K-12 and the sequence of its rfb gene cluster. J. Bacteriol. **176:**4144-4156.
15. **Kropinski, AM, Mazzocco, A, Waddell, TE, Lingohr, E, Johnson, RP.** 2009. Enumeration of bacteriophages by double agar overlay plaque assay. Methods Mol. Biol. **501:**69.
16. **Abedon, ST.** 1992. Lysis of lysis-inhibited bacteriophage T4-infected cells. J. Bacteriol. **174:**8073-8080.
17. **Anderson, B, Rashid, MH, Carter, C, Pasternack, G, Rajanna, C, Revazishvili, T, Dean, T, Senecal, A, Sulakvelidze, A.** 2011. Enumeration of bacteriophage particles: Comparative analysis of the traditional plaque assay and real-time QPCR- and NanoSight-based assays. Bacteriophage. **1:**86-93. doi: 10.4161/bact.1.2.15456.
18. **Edelman, DC, Barletta, J.** 2003. Real-time PCR provides improved detection and titer determination of bacteriophage. BioTechniques. **35:**368.
19. **Bremer, M, Doerge, RW.** 2010. Statistics at the bench: a step-by-step handbook for biologists. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
20. **Seed, KD.** 2015. Battling Phages: How Bacteria Defend against Viral Attack: e1004847. PLoS Pathogens. **11:**.
21. **Guo, H, Yi, W, Shao, J, Lu, Y, Zhang, W, Song, J, Wang, PG.** 2005. Molecular Analysis of the O-Antigen Gene Cluster of Escherichia coli O86:B7 and Characterization of the Chain Length Determinant Gene (wzz). Appl. Environ. Microbiol. **71:**7995-8001.

22. **Voorhout, WF, Leunissen-Bijvelt, JJ, Leunissen, JL, Verkleij, AJ.** 1986. Steric hindrance in immunolabelling. J. Microsc. **141:**303.
23. **Kemp, P, Garcia, LR, Molineux, IJ.** 2005. Changes in bacteriophage T7 virion structure at the initiation of infection. Virology. **340:**307-317.