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Rescue of O16 Antigen expression in *E. coli* strain MG1655 Prevents Adsorption of T4 Bacteriophage

Justine Dimou, Jolin Lu, Shannon Pflueger, Eric Toombs

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

SUMMARY O antigen is the outermost component of lipopolysaccharide and is expressed on the surface of Gram-negative bacteria. The O antigen O16 in *Escherichia coli* K-12 has been associated with resistance to T4 bacteriophage infection and its biosynthesis requires functional components encoded by the *rfb* locus, including *wbbL* which encodes a rhamnose transferase. Previous experiments have demonstrated that the presence of functional *wbbL* conveys *E. coli* strains complete resistance to T4 bacteriophage mediated lysis, while disrupted *wbbL* leaves them susceptible. However, the mechanism through which this resistance occurs is still unclear. Due to O16 antigen being a surface molecule, we hypothesized that the presence of *wbbL* prevents T4 bacteriophage adsorption onto the surface of *E. coli* K-12. In this study, we investigated T4 bacteriophage adsorption ability via a chloroform-based adsorption assay on both *E. coli* MG1655 and DFB1655 L9. These are two completely isogenic strains differing only in *wbbL* functionality, with MG1655 unable to produce O16. We found that the number of unadsorbed T4 bacteriophage decreased to 17% of the original concentration over 15 minutes when incubated with MG1655, presenting an adsorption rate constant of 2.8 x 10^{-9} mL/min(cfu). When incubated with DFB1655 L9 however, the number of unadsorbed T4 bacteriophage did not decrease even after 25 minutes of incubation, indicating that T4 bacteriophage can adsorb to MG1655 but not to DFB1655 L9. Since these two strains are isogenic except for *wbbL,* we conclude that the presence of functional *wbbL* in *E. coli* strain DFB1655 L9 prevents T4 bacteriophage adsorption.

INTRODUCTION

Lipopolysaccharide (LPS) is present on the surface of most gram-negative bacteria and can contain a molecule known as O antigen, which sits at the outermost part of LPS and has been classified into more than 180 different variants (1). Some bacteriophages use these O antigens as host receptors and are highly specific to the type of O antigen variant (2). In contrast, there are other bacteriophages that only target bacterial cells which lack O antigen (2). Since bacteriophages have these specificities, any modification to the required receptor or receptor environment may inhibit phage infection (2).

The biosynthesis of O antigen O16 in *E. coli* K-12 requires functional components encoded by the *rfb* locus, specifically *wbbL* encoding a rhamnose transferase (3). The *E. coli* MG1655 strain lacks O16 due to an IS5 insertion in *wbbL*, while the *E. coli* DFB1655 L9 strain expresses O16 due to the regeneration of functional *wbbL* (3). Other than this IS5 insertion, the two strains are genetically identical (3). Using these two strains, previous experiments demonstrated that the presence of *wbbL* conveys DFB1655 L9 complete resistance to T4 bacteriophage mediated lysis, while disrupted *wbbL* leaves MG1655 susceptible (4).

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

In this paper we discuss bacteriophage adsorption, binding, and attachment (binding and attachment are also commonly known as reversible binding and irreversible binding respectively). Adsorption refers to the complete three-step process which results in the phage being irreversibly bound to the host cell (2). The first step is initial contact with the host, the second step is binding, and the third step is attachment (2). Binding is reversible, so if the phage is not bound to the correct receptor(s) it may desorb and continue until it finds the correct receptor(s) (2). Attachment is irreversible, and once it happens it allows for insertion of the phage genome into the host (2).

Many attempts have been made to better understand the mechanism of T4 phage resistance in DFB1655 L9. Initially, it was hypothesized that the presence of O16 antigen on the surface of cells sterically blocks virus adsorption (5). Wachtel et al. conducted an adsorption assay by using centrifugation and qPCR to measure the phage concentration in supernatants from cells exposed to bacteriophage just long enough to allow attachment (5). Their results showed more viral DNA in supernatants from DFB1655 L9 than MG1655, supporting the steric hindrance model (5). However, replication of this assay by Lee et al. did not observe a significant difference in adsorption between the same two strains (6). Instead, they proposed that phage particles may be binding to cells but are either unable to inject their DNA or unable to replicate intracellularly (6). The most recent study by Biparva et al. managed to image the adsorption of T4 bacteriophage by electron microscopy (N. Biparva, A. Ghazizadeh, T. Hoang, and S. Sun, submitted for publication). T4 phage was visually observed bound to MG1655 cells but not DFB1655 L9. This supports the decreased adsorption hypothesis, but their results were preliminary due to a limited number of samples.

We hypothesized that the absence of functional *wbbL* in MG1655 allows for T4 phage adsorption, while in DFB1655 L9 the presence of functional *wbbL* prevents T4 phage adsorption. To further investigate T4 phage adsorption to MG1655 compared to DFB1655 L9 we used an assay to measure the rate of irreversible attachment in both *E. coli* K12 strains. This assay consists of time course sampling of MG1655 and DFB1655 L9 cultures after T4 infection (7). Chloroform is then added under cold conditions to kill the bacterial cells and abolish reversible binding (7). This results in the separation of T4 phage particles that did not irreversibly attach to cells and the ability to quantify them as plaque forming units per milliliter (pfu/mL). In this study we show that the attachment rate constant for T4 phage to MG1655 is 2.8×10^{-9} mL/min(cfu), while it is 0 mL/min(cfu) for DFB1655 L9. Our results confirm that T4 bacteriophage does not irreversibly attach to DFB1655 L9 due to the presence of a functional *wbbL*.

METHODS AND MATERIALS

Bacterial and Bacteriophage Strains. Bacterial strains *E. coli* MG1655 and DFB1655 L9 as well as T4 bacteriophage were obtained from the lab stocks in the Department of Microbiology and Immunology at the University of British Columbia.

PCR and gel electrophoresis. Colony PCR was performed for three colonies each of MG1655 and DFB1655 L9 strains. Primers designed by Browning et al. were used to amplify both rescued *wbbL* in DFB1655 L9 and *wbbL* containing the insert sequence in MG1655 (3). The identity of the T4 bacteriophage stock was confirmed by amplification of *gp23* using primers from Chiu et al. (4). The PCR products were run on a 1% agarose gel in 0.5X TAE with a 1 Kb Plus DNA ladder from Thermo Fisher Scientific and visualized with SYBR Safe.

T4 Bacteriophage Propagation. *E. coli* MG1655 was grown overnight at 37°C in LB broth. Overnight culture was diluted to an OD_{600} of 0.5 and $CaCl_2$ was added to a concentration of 1 mM. T4 bacteriophage stock was added to the cells and the mixture was incubated at 37°C, shaking at 200 rpm for approximately 4 hours until lysis of MG1655 was visible. Lysate was then split into glass tubes, chloroform was added to kill any remaining cells, and samples were refrigerated overnight. Lysates were then combined and centrifuged at 8000 x g for 5 minutes to pellet cells and debris. Supernatant containing purified T4 bacteriophage was filter sterilized through a 45 µm filter and then stored at 4°C.

Determination of Bacteriophage Stock Titer. Serial dilutions of T4 bacteriophage stock were prepared ranging from 10^{-3} to 10^{-8}. Underlay agar was prepared with tryptone, yeast extract, NaCl and a 15g/L concentration of agar. Overlay agar was prepared with the same components but with an agar concentration of 5g/L. Underlay and overlay agar solutions were supplemented with $CaCl_2$ to a concentration of 1mM to facilitate T4 infection. 100µL each of a phage dilution and 100µL of an overnight MG1655 culture were added to 3 mL of warm overlay agar. After mixing briefly, the overlay mixture was poured onto a dry underlay agar plate and allowed to dry before incubating overnight at 37°C. Each dilution was performed in duplicate and concentration of T4 bacteriophage was then calculated by plaque counting.

T4 Bacteriophage Adsorption Assay. The adsorption assay is as described by Kropinski (7). Overnight cultures of *E. coli* MG1655 and DFB1655 L9 were diluted 1:100 and incubated in the presence of 1mM $CaCl_2$ until they reached an OD_{600} of ~0.1. 1mL of T4 phage at a concentration of $2x10^5$ pfu/mL was added to flasks with 9 mL of log phase MG1655 or DFB1655 L9 cells at 37°C. A sample was taken every minute of incubation up to 10 minutes: 50µL were removed and added to a tube containing 1mL of LB with 5% chloroform. Samples were then vortexed for 10 seconds and placed on ice. Two additional time points of 15 minutes and 25 minutes after T4 phage addition were also sampled. Chloroform separated out of solution after 1-2 minutes on ice, after which the chloroform-free supernatant was collected. Once samples for all time points were taken, phage concentrations were quantified by the same soft agar overlay method used to quantify the initial phage titer. Each time point was performed in duplicate. Leftover bacterial culture from the initial inoculation was kept on ice throughout the experiment and then spread-plated on underlay plates at serial dilutions of 10^{-5} and 10^{-6} to determine bacterial concentration. After incubating overnight at 37°C, plaques were counted to determine T4 adsorption and bacterial colonies were counted to determine bacterial concentration.

Calculation of Attachment Rate Constant. In order to quantify the rate of attachment of bacteriophage to bacterial cells at any given time point, we can apply the equation

$$\frac{d[P]}{dt} = -k([B])([P]_t - [P]_e) \quad (1)$$

where (d[P]/dt) is the rate of change of free phage concentration, k is the rate constant, [B] is the bacterial concentration, $[P]_t$ is the concentration of free phage at time t, and $[P]_e$ is the concentration of free phage at equilibrium (8). Given a multiplicity of infection (MOI) of 5.1 x 10^{-4} and 7.7 x 10^{-4} for MG1655 and DFB1655 L9 respectively (Table S1, S2), we can safely assume that every phage will attach to a cell if given enough time and that $[P]_e$ is equal to 0. As well, because 1mL of phage was added to 9mL of bacterial culture to begin the adsorption assay, 90% of the determined bacterial concentration must be used for [B].
Since the MOI is very low, [B] can be treated as a constant relative to [P], so integrating and solving for k gives

$$k = \frac{2.3}{t[B]} log \frac{[P]_0}{[P]_t} \quad (2)$$

where $[P]_0$ is the initial concentration of bacteriophage and t is the time interval from $[P]_0$ to $[P]_t$ (7). The determined value of k can then be substituted back into Eq. 1 to give the phage attachment rate in pfu/mL for any given $[P]_t$.

RESULTS

PCR of *E. coli* K12 strains confirmed disrupted *wbbL* in MG1655 and rescued *wbbL* in DFB1655 L9. To confirm the identity of MG1655 and DFB1655 L9, colony PCR was conducted to amplify *wbbL*. MG1655 contains a disruptive IS5 element in *wbbL* and has an expected size of 1994 base pairs. DFB1655 L9 contains a reintroduced functional *wbbL* by use of pET20b/*wbbL* and has an expected size of 799 base pairs (3). PCR products from six separate colonies showed successful amplification (Fig. 1A) for MG1655 (lanes 2-4) and DFB1655 L9 (lanes 5-7). No individual negative controls were used, since both strains used

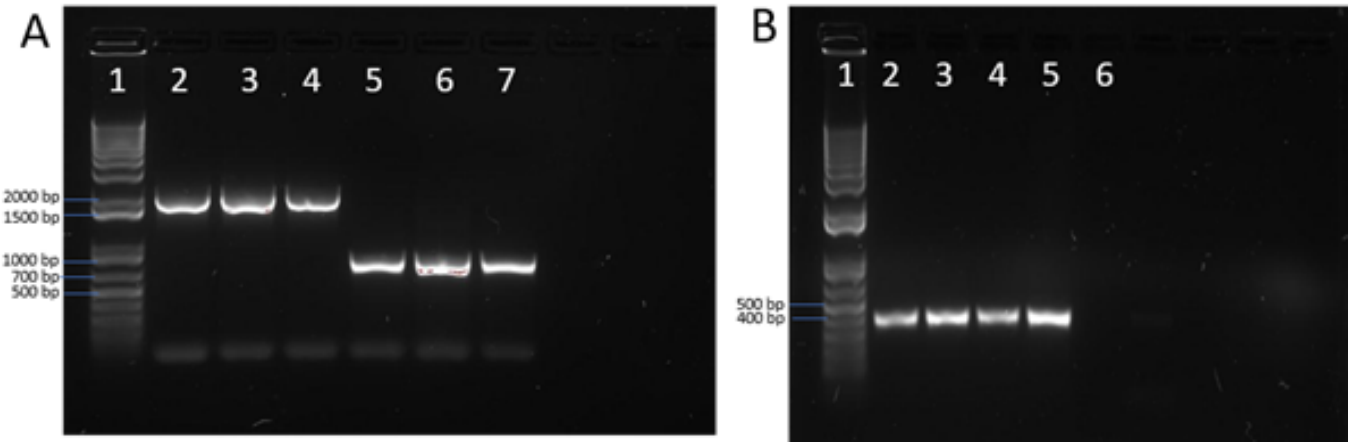

FIG. 1 Confirmation of *E. coli* strains and T4 bacteriophage via PCR and gel electrophoresis. (A) *wbbL* was amplified via PCR from strains MG1655 (lanes 2-4) and DFB1655 L9 (lanes 5-7). PCR products were run on a 1% agarose gel. The expected band sizes are 1994bp for MG1655 and 799bp for DFB1655 L9. (B) *gp23* was amplified via PCR and run on a 1% agarose gel. Lanes 2-5 contained T4 bacteriophage which has an anticipated band size of 398bp.

the same primers each strain can act as a negative control for the other. These results verify that the *E. coli* strains used present the correct expected genetic elements.

Titer of T4 bacteriophage stock. A double agar overlay plaque assay was used to determine the concentration of the T4 bacteriophage stock. Various dilutions of bacteriophage were mixed with *E. coli* MG1655, plated, and incubated overnight at 37°C. A titer of 1.01×10^{10} pfu/mL was determined.

PCR confirmed T4 bacteriophage identity through *gp23* amplification. To confirm the identity of the T4 bacteriophage stock, PCR amplification of *gp23* was performed. *gp23* codes for the major capsid protein and has an expected band size of 398 base pairs. T4 bacteriophage was added to the PCR mix in varying concentrations to maximize potential for successful amplification (Fig. 1B, lanes 2-5). Amplification of *gp23* was observed at all T4 phage concentrations verifying the identity of the T4 bacteriophage.

DFB1655 L9 is resistant to T4 bacteriophage infection. To confirm that DFB1655 L9 is resistant to T4 bacteriophage infection, double agar overlay assays were performed and plated in duplicate (Fig. S1). T4 bacteriophage at 2×10^5 pfu/mL was combined with MG1655 or DFB1655 L9 in overlay medium and was spread across the underlay plate. As expected, plates with MG1655 showed numerous plaques whereas plates with DFB1655 L9 showed no plaques. These results support the observation that MG1655 is susceptible, and DFB1655 L9 is resistant to T4 lysis.

Adsorption of T4 bacteriophage is inhibited in DFB1655 L9 but not MG1655. To investigate the differences in T4 viral adsorption, the adsorption assay outlined in the methods above was used. The amount of free T4 phage in supernatants was measured every minute for 10 minutes immediately after infection of MG1655 and DFB1655 L9 cells. Two other time points at 15 minutes and 25 minutes were also measured with minutes 1 to 10 being plated in duplicate. Bacteriophage was incubated with sterile LB and sampled in duplicate after 10 minutes to act as a control and as time point t = 0 minutes (Fig. 2). The control was sampled at the end of the assay to account for any natural phage degradation that may have occurred over time. The amount of phage present in the bacteria-free negative controls after 10 minutes was consistent with the amount initially added confirming that incubation and chloroform treatment did not alter the amount of functional T4 phage. A negative trend in free phage present is seen when incubated with MG1655, reaching 17% of the original concentration after 15 minutes (Fig. 2A). Using Eq. 2 in the Materials and Methods, and the phage concentrations at t = 0 and t = 15 (Table S1), we find an attachment rate constant of

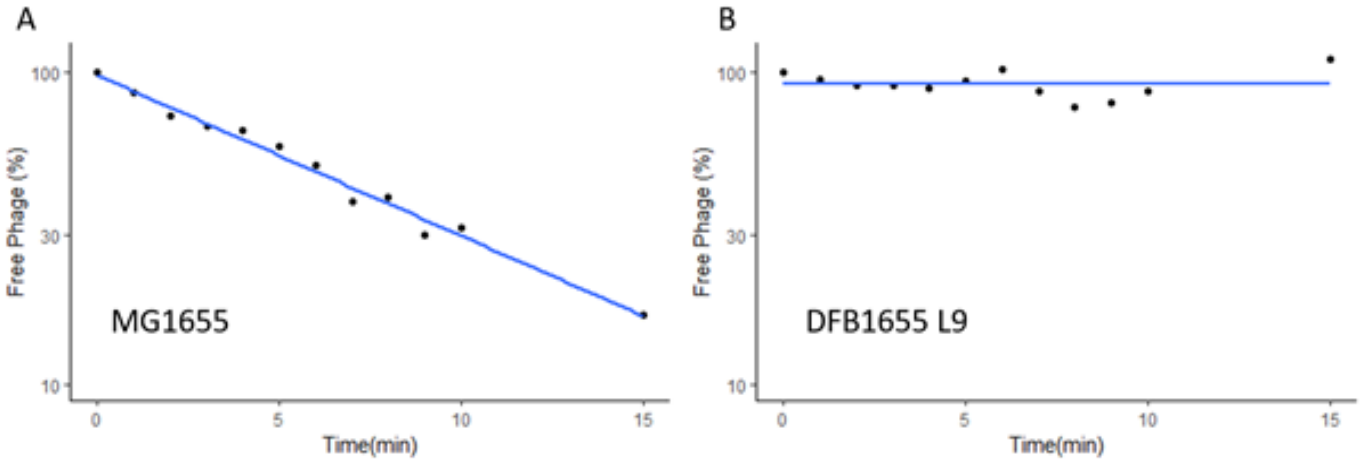

FIG. 2 Double overlay adsorption assay measuring free phage present in supernatant. Free phage present after T4 bacteriophage adsorption assay with MG1655 strain (A) and DFB1655 L9 strain (B) at time points 1-10 minutes, and 15 minutes. The y-axes have been scaled logarithmically.

2.8 x 10^{-9} mL/min(cfu). In contrast, the quantity of unadsorbed phage remained consistent over time when incubated with DFB1655 L9 (Fig. 2B). By once again using Eq. 2, and by estimating P_0 to be equal to P_t by the line of best fit in Fig 2., we find the attachment rate constant to be 0 mL/min(cfu). This means that k will be equal to zero in Eq. 1 and the attachment rate of the phage at any given time point will be zero regardless of the other variables in the equation. This provides evidence that T4 phage cannot irreversibly attach to DFB1655 L9. The additional 25-minute time point showed that after 25 minutes the quantity of phage in DFB1655 L9 supernatants was still unchanged (Fig. S2). However, after 25 minutes with MG1655, T4-mediated lysis occurred and the number of phage particles measured in the supernatant largely increased, exceeding the quantifiable range of the plaque assay (Fig. S2). This result emphasizes that T4 phage cannot attach to DFB1655 L9, even if given more time. In conclusion, the quantity of free T4 bacteriophage decreased in supernatants from MG1655 but stayed constant in supernatants from DFB1655 L9, suggesting that T4 phage adsorption is inhibited in DFB1655 L9 but not MG1655

DISCUSSION

The objective of this study was to provide more clarity on the mechanism of T4 bacteriophage resistance caused by functional *wbbL* in *E. coli* K12, specifically investigating viral adsorption. For cells to be infected, T4 bacteriophage must encounter cells, bind in a reversible manner, then attach irreversibly to inject their DNA (2). We quantified adsorption by measuring the amount of free functional T4 phage that remained in the supernatant after exposure to MG1655 or DFB1655 L9 and subsequent chloroform treatment. Since T4 bacteriophage does not contain any lipids, it is unaffected by chloroform and the amount of phage can be accurately quantified (9,10). After exposure to MG1655, there were progressively fewer T4 phage present in the supernatant over 15 minutes. At 25 minutes after infection, the number of phage particles in the supernatant dramatically increased indicating phage-induced lysis had occurred somewhere between 15 and 25 minutes of incubation. Contrarily, the amount of free T4 phage stayed the same after exposure to DFB1655 L9 for the entire 25 minutes after infection. Since MG1655 and DFB1655 L9 are isogenic and only differ in their functionality of *wbbL*, we can interpret that the presence of functional *wbbL* prevents T4 phage from fully adsorbing and inserting its genome into the cell.

Since adsorption occurs sequentially in three steps: initial contact, reversible binding, and irreversible attachment, *wbbL* must at least be inhibiting irreversible attachment (2). It is important to note that we cannot make any conclusions about initial contact or reversible binding since it is possible T4 phage could still make contact and reversibly bind to DFB1655 L9. To mitigate any reversible binding that may occur to dead cells or dead cell debris during

chloroform treatment, cold conditions were used to inhibit reversible binding (7). Tubes of infected cultures were placed on ice after chloroform treatment resulting in any reversibly bound phage being released into the supernatant.

Phage resistance by bacteria has been previously classified into three categories: blocking of phage receptors, production of extracellular matrix, and production of competitive inhibitors (11). Therefore, it is possible that the large O16 polysaccharide created by functional *wbbL* in DBF1655 L9 directly inhibits full T4 phage adsorption. Though some bacteriophages, such as *Salmonella phage P22*, recognize O antigens as receptors, the irreversible attachment of T4 phage on *E. coli* K-12 strains involves the attachment of long tail fibers onto the outer membrane and short tail fibers onto the LPS core region, particularly the heptose moiety (11, 12). Thus, it is possible that the LPS core receptor and outer membrane are inaccessible to the tail fibers required for irreversible attachment. It may also be possible that O16 antigen prevents reversible binding from occurring, which would prevent the phage from coming close enough to the cell to even attempt to irreversibly attach.

For determining the concentration of phage in supernatants, plaque assays are potentially more reliable than the qPCR methods done previously (5,6). qPCR measurements are indirect and could be subject to error from DNA contamination or free-floating DNA from destroyed or incomplete phage. For some types of phage, concentration estimates by qPCR were observed to be inflated compared to plaque assays (13). The benefit of using the double agar overlay plaque assay is that it directly measures the number of whole functional phage. In these assays, each plaque that forms theoretically corresponds to one functional phage particle, which is simple to interpret.

The adsorption assay employed was used to reliably measure how the presence of *wbbL* affects the ability of T4 phage to adsorb to *E. coli* K-12. We provide evidence that T4 bacteriophage readily attaches to MG1655, which lacks functional *wbbL,* but does not attach to DFB1655 L9, which is isogenic to MG1655 but contains functional *wbbL*. In conclusion, our data suggest that the presence of functional *wbbL* in *E. coli* K-12 prevents phage from irreversibly attaching to cells. We cannot say whether or not reversible binding is also affected, but our findings bring clarity to previous experiments and add evidence that the mechanism responsible for *wbbL* acquired resistance is the inhibition of complete T4 adsorption.

Future Directions While this project specifically investigated whether the presence of O16 antigen inhibits T4 bacteriophage adsorption to *E. coli*, *E. coli* express many different types of O antigens on their surface (1). Future research could investigate the role of other types of O antigen in T4 bacteriophage resistance by knocking out and rescuing the genes encoding for a specific type of O antigen. Similarly, there are different types of bacteriophages which target various parts of LPS on other bacterial species (2). Future experiments could investigate which O antigens will confer resistance to which bacteriophages.

Further, as mentioned previously, this adsorption assay cannot determine if *wbbL* also inhibits the reversible binding of T4 phage. An assay to differentiate between the two adsorption steps of reversible binding and irreversible attachment would further elucidate the mechanism by which *wbbL* stops T4 adsorption.

Ideally, future experiments would also phenotypically verify that O16 antigen is expressed on the surface of DFB1655 L9, as it was only predicted here through the presence of functional *wbbL*.

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