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Soluble O^{16}-antigen LPS complex delays T4 bacteriophage adsorption to *Escherichia coli* K-12 MG1655

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SUMMARY MG1655 is an *Escherichia coli* K-12 strain that does not express O-antigen, a distal sugar-component of lipopolysaccharide (LPS) on Gram-negative bacteria. DFB1655 L9 is an isogenic strain of MG1655 with O^{16}-antigen expression restored. Studies have shown that DFB1655 L9 is resistant to T4 bacteriophage infection, whereas MG1655 is susceptible. How O^{16}-antigen confers resistance to T4 is unknown. Elucidating this resistance mechanism will provide insight into the functional role of O-antigen. Here, we investigated the effects of soluble O^{16}-antigen on T4 adsorption to *E. coli* K-12 by performing a time series adsorption assay of T4 to MG1655 using T4 pre-treated with LPS with or without O^{16}-antigen. The free phage concentration in the supernatant, representing the unadsorbed phage, was quantified via double agar overlay plaque assays. We observed a similar trend between the control group and the treatment group of T4 incubated with LPS without O^{16}-antigen, in which free phage concentration decreased rapidly to 3.10% and 2.99% at t = 5 minutes, respectively. When T4 was incubated with LPS with O^{16}-antigen, a delayed decrease of free phage concentration was observed, with 17% of the initial free phage concentration remaining at t = 5 minutes. However, eventually phage in the supernatant of all groups depleted. Therefore, we conclude that soluble O^{16}-antigen LPS complex was able to delay T4 adsorption to MG1655 but did not confer long-term resistance. This provides evidence that soluble O^{16}-antigen likely affects T4 bacteriophage infectivity through reversible interactions and delays its rate of adsorption to the cell surface.

INTRODUCTION

Lipopolysaccharide (LPS) present on the outer membrane of Gram-negative bacteria, such as *Escherichia coli,* is known to serve as a receptor for some bacteriophages, including T4 (1). In order to infect a host cell, T4 bacteriophage must adsorb to the cell membrane in a process that involves the following steps: initial phage contact, reversible binding, and then irreversible attachment (2). Reversible binding is mediated by long-tail fibers on T4 phage, while irreversible attachment involves short tail fibers (3). Previous studies have shown that this early stage of infection is supported by both intact LPS and OmpC (an osmoregulator) (2, 4). Furthermore, variants of *E. coli* with abnormal OmpC expression or mutated LPS have exhibited resistance to T4 infection (2, 5-12). *E. coli* K-12 strain MG1655 does not express O-antigen, a repeating polysaccharide on the core of LPS, and is susceptible to T4 infection (6-12). *E. coli* K-12 strain DFB1655 L9 is isogenic with MG1655 with restored O^{16}-antigen expression and has demonstrated resistance to T4 infection (6-13).

There is an ongoing investigation into the role of O-antigen during T4 phage infection and the potential mechanism of resistance it confers. Using quantitative polymerase chain reaction (qPCR) methods, double agar overlay plaque assays, and electron microscopy (EM) visualization, several studies to date support that the presence of O-antigen inhibits T4

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adsorption (7, 9-11). Dimou *et al.* found that incubating resistant DFB1655 L9 strain (which expresses O^{16}-antigen) with T4 phage resulted in higher phage titers remaining in the supernatant compared to strain MG1655, suggesting that T4 was not able to adsorb and enter the host cell (7, 9). Electron microscopy imaging appeared to show phage embedded in the outemembrane of resistant strains, again supporting that infection is inhibited at the adsorption stage (10, 11). Taken together, these findings may suggest that T4 phage interacts with surface-bound O-antigen at the cell membrane, which disallows it from neither entering nor detaching to find another receptor, effectively preventing infection. Another model that could explain the resistant phenotype of the DFB1655 L9 strain is that soluble factors of LPS, such as components of O-antigen that may be released from the cell membrane into the culture supernatant, potentially bind to the phage, and subsequently inactivate it via chemical interactions (6, 14). This model would suggest that free O-antigen in the environment is sufficient for conferring T4 resistance, and may be a promising prospect for future research.

The proposed model that soluble LPS/O-antigen moieties may be inactivating phage in the supernatant was previously investigated by Chiu *et al*. (6). In their study, they treated T4 phage with culture supernatant of the resistant DFB1655 L9 strain before inoculating both resistant and susceptible cells. They observed no difference in infectivity, implying that resistance is not attributable to secreted factors. However, their study was limited by the fact that the supernatant was not characterized nor specific for O-antigen. It is possible that the amount of O-antigen or LPS factors in the supernatant was not sufficient for conferring resistance. A different study found that T4 phage adsorbed to free LPS, which was able to neutralize the phage (15). To validate these contradicting observations and further investigate this potential model of resistance, we pre-treated T4 phage with purified LPS/O^{16}-antigen prior to incubation with susceptible cells in order to investigate the effect of soluble O^{16}-antigen on T4 adsorption to *E. coli* K-12.

We hypothesized that treatment of T4 phage with LPS isolated from cell lysates of the DFB1655 L9 strain would inhibit its adsorption to susceptible MG1655 cell surfaces. This would test the model that soluble/free O^{16}-antigen may be inactivating phage in the environment and inhibiting its ability to adsorb to the cell surface.

METHODS AND MATERIALS

Bacteria and bacteriophage strains. *E. coli* K-12 MG1655, *E. coli* K-12 DFB1655 L9, and T4 bacteriophage strains were obtained from the Department of Microbiology and Immunology at the University of British Columbia.

Identity check via PCR and gel electrophoresis. Genomic DNA of both MG1655 and DFB1655 L9 strains were extracted using EZ-10 Spin Column Genomic DNA Miniprep Kit (Bio Basic). PCR was then performed on the genomic DNA from both strains with Platinum® *Taq* DNA Polymerase (Invitrogen) and primers designed by Browning *et al.* targeting *wbbL* gene (Table S1) (13). PCR was also performed on T4 bacteriophage to confirm its identity using primers designed by Chiu *et al.* targeting the *gp23* gene (Table S1) (6). The T100™ Thermal Cycle (Bio-Rad) was programmed for a 5-minute initial denaturation step at 95°C, followed by 30 cycles of denaturation for 30 seconds at 95°C. At each cycle, a 45-second annealing phase was programmed at 55°C, and a 2.5-minute extension phase at 75°C. The PCR products were run on a 1% agarose gel with O'GeneRuler DNA ladder (Fermentas) for both bacterial strains alongside a 100 base pair (bp) DNA ladder (Invitrogen) for T4 bacteriophage in 1x TAE buffer at 120 V for 15 minutes and 80 V for 35 minutes. The gel was visualized with SYBR Safe DNA Gel Stain (Invitrogen) and imaged with ChemiDoc MP Imaging System (Bio-Rad).

Confirm T4 bacteriophage functionality via lysis assay. Lawns of MG1655 and DFB1655 L9 were swabbed onto LB and LB-kanamycin plates (50 µg/mL), respectively. T4 bacteriophage stock was briefly vortexed for 10-20 seconds and 8 µL droplets of T4 were dispensed onto the surface of the bacterial lawns, then allowed to completely dry by a flame. The plates were incubated for 16 hours at 37°C, then evaluated for plaques.

LPS extraction from MG1655 and DFB1655 L9. Lawns of MG1655 and DFB1655 L9 were grown overnight at 37°C on LB and LB-kanamycin plates (50 µg/mL), respectively. LPS was extracted from MG1655 and DFB1655 L9 following the LPS Extraction Kit (ab239718) manufacturer instructions (Abcam). The manufacturer protocol was modified to include 3 cycles of 20 seconds beating using the FastPrep-24™ bead grinder (MP), rather than 3 cycles of 20 seconds of sonication. Extracted LPS was stored at 4°C. Absence of O^{16}-antigen in MG1655 and presence of O^{16}-antigen in DFB1655 L9 LPS extracts were confirmed through SDS-PAGE and silver staining. Extracts were vigorously vortexed for 2 minutes, then diluted 1:10 with endotoxin free water. 10 µL of samples were prepared per well using a 2x Laemmli sample buffer (Bio-Rad) onto a 4-20% Mini-PROTEAN® TGX™ Precast Protein Gel (Bio-Rad). The PageRuler™ Prestained Protein Ladder (Thermo Scientific) was also loaded to determine molecular weights. The gel was run at 205 V for 30 minutes, then subsequently silver stained following SilverQuest™ Silver Staining Kit instructions (Invitrogen). Immediately after, the stained gel was visualized using the ChemiDoc MP Imaging System (Bio-Rad).

T4 propagation. An overnight culture of E. coli MG1655 was incubated in LB broth at 37°C. The next morning, the culture was diluted down to an OD600 below 0.5. CaCl2 was added to achieve a final concentration of 1 mM. 1 mL of T4 phage stock was added to the culture and incubated at 37°C on a shaking platform at 200 rpm for 4 hours until a visible clearing of culture indicated lysis, as confirmed by a decrease in the OD600 reading. The lysate was aliquoted into multiple glass test tubes, and chloroform was added in a 1:50 ratio of chloroform to culture in order to kill any remaining cells. The lysate was stored overnight at 4°C. The next day, the lysate was centrifuged at 8000 x g for 5 minutes to pellet cell debris. Additional centrifugation steps of 8000 x g for 5 minutes followed by 3000 g for 10 minutes were taken to improve cell pelleting. The supernatant was harvested and filtered through a 45 µM filter, then stored at 4°C.

T4 stock titer. T4 stock titer was determined via double agar overlay plaque assays. The 1.5% underlay agar was prepared with tryptone, yeast extract, NaCl, and 15 g/L of agar supplemented with 1 mM $CaCl_2$. The 0.7% overlay agar was prepared with 7 g/L of agar and with other components kept the same. A serial dilution of purified T4 stock ranging from 10^{-3} to 10^{-8} with 1 mM $CaCl_2$ LB broth was prepared. An overnight culture of MG1655 was grown in LB broth at 37°C. 100 µL of MG1655 culture and 100 µL of T4 dilution were added to 3 mL of warm 0.7% overlay agar, then the mixture was poured on top of the solidified underlay layer. All plates were dried and solidified under a flame, then incubated at 37°C overnight. The T4 stock titer (PFU/mL) was determined from the number of plaques per countable plate (30 - 300 colonies).

Adsorption assay of T4 bacteriophage incubated with MG1655 and DFB1655 L9 LPS extracts. The protocol of the adsorption assay was adapted from Kropinski and Dimou *et al.* (9, 16). MG1655 culture was grown overnight at 37°C in LB broth. The next day, the overnight culture was diluted to an OD_{600} of ~0.05, then incubated at 37°C with 1 mM of $CaCl_2$ until reaching an OD_{600} between 0.1 and 0.2 to ensure that bacterial cells were at log phase. This OD_{600} reading was used to determine the initial bacterial concentration. 200 µL of T4 was incubated with 800 mL of either MG1655 or DFB1655 L9 LPS for 2 hours, reaching a final phage concentration between 1 x 10^5 to 3 x 10^5 PFU/mL. 1 mL of either T4 incubated with MG1655 or DFB1655 L9 LPS was added to a flask with 9 mL of log phase MG1655. As a control, 1 mL of 2.85 x 10^5 PFU/mL T4 was added to 9 mL of log phase MG1655. 50 µL of solution was removed from each flask and added to 1 mL of chilled LB containing 5% chloroform every minute up to 10 minutes, at 15 minutes, and at 25 minutes. Solutions were vortexed for 10 seconds, and immediately placed on ice to allow for the chloroform to separate out of the solution. Free phage concentrations at each time point were quantified using double agar overlay assays. The underlay and overlay agar consisted of 1.5% and 0.7% LB-agar respectively, both supplemented with 1 mM of $CaCl_2$ to facilitate T4 infection. 100 µL of chloroform-free supernatant was collected from each time point, mixed with 100 µL of the MG1655 culture and 3 mL of warm overlay agar, then poured onto dry

underlay agar plates and allowed to solidify. Plates were dried for approximately 20 minutes to ensure no moisture would be trapped when closing the lids. Plates were incubated for 16 hours at 37°C, then plaques were counted to determine free phage concentration, and thus T4 adsorption. 2 biological replicates (n = 2) were conducted for this assay.

RESULTS

PCR confirmed the identity of MG1655 and DFB1655 L9 strains through *wbbL* amplification. To characterize MG1655 and DFB1655 L9, PCR was performed on both strains using primers flanking the *wbbL* gene. MG1655 is expected to produce a band size of 1994 base pairs because the *wbbL* gene is disrupted by a small transposable insertion sequence (13). DFB1655 L9 contains an intact *wbbL* gene restored by pJP5603/*wbbL* conjugative suicide plasmid, and therefore it has an expected size of 799 bases pairs (13). PCR amplification on each strain resulted in products corresponding to their expected sizes, where we observed a PCR product at 2 kbp for MG1655 and a PCR product at 800 bp for DFB1655 L9 (Fig. 1A). In conclusion, the identity of both strains was verified based on the presence of expected *wbbL* genotypes.

PC R confirmed the identity of T4 bacteriophage through *gp23* amplification. To confirm T4 bacteriophage identity, PCR was performed with primers flanking the *gp23* gene. The T4 bacteriophage gene *gp23* encodes one of the major capsid proteins, and was chosen due to its high transcription levels (17). The PCR amplification of *gp23* has an expected band size of 398 base pairs. PCR amplification on T4 showed specificity for the desired products, where we observed products at 400 bp (Fig. 1B). Negative control was run in parallel with UltraPure water and there was no production of nonspecific PCR products and/or primer-dimers (Fig. 1B). Collectively, the PCR assay confirmed the identity of T4 bacteriophage stock.

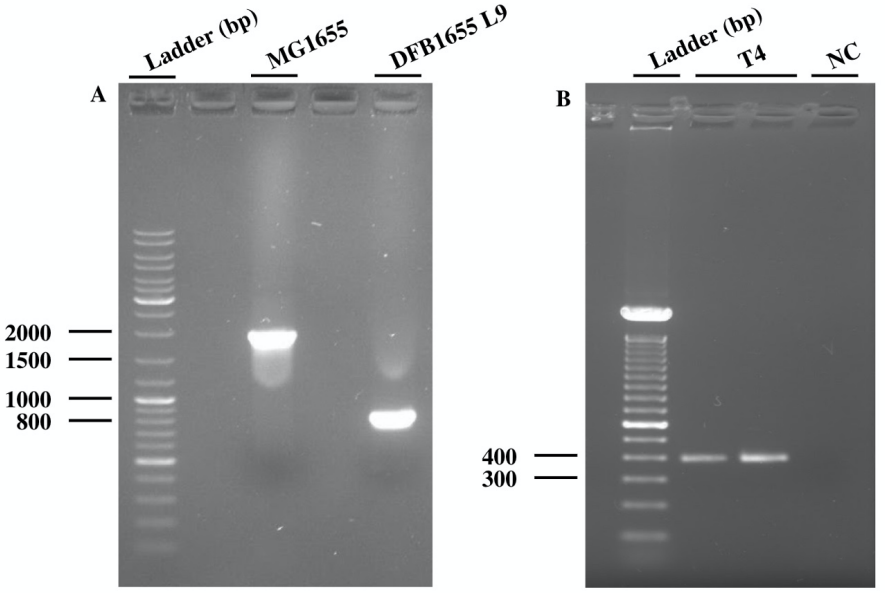

FIG. 1 Confirming the identity of *E. coli* strains MG1655, DFB1655 L9, and T4 bacteriophage through PCR and gel electrophoresis. (A) PCR amplification of *wbbL* was performed on the genomic DNA extracted from both MG1655 and DFB1655 L9 strains. The PCR products run on 1% agarose gel were shown to have the expected band sizes of 1994 bp for MG1655 and 799 bp for DFB1655 L9. (B) PCR amplification of *gp23* was performed on T4 bacteriophage. The PCR product run on 1% agarose gel is shown to have the anticipated band size of 398bp. NC indicates a negative control consisting of UltraPure water instead of DNA.

MG1655 was susceptible to T4 bacteriophage infection whereas DFB1655 L9 was resistant. To confirm that MG1655 was susceptible to and DFB1655 L9 was resistant to T4 bacteriophage infection, a lysis assay was performed. Three 8 µL T4 bacteriophage droplets were dispensed on MG1655 and DFB1655 L9 lawns. Plaques were observed at droplet locations on the MG1655 lawn, indicating that the bacteriophage lysed the cells (Fig. 2). In contrast, no plaques were observed on the DFB1655 L9 lawn, indicating that the bacteriophage did not lyse the cells and that DFB1655 L9 was resistant to T4 bacteriophage infection (Fig. 2). Results from this lysis assay confirmed the activity of our T4 bacteriophage stock and the identity of both *E. coli* substrains based on their susceptibility to T4 bacteriophage.

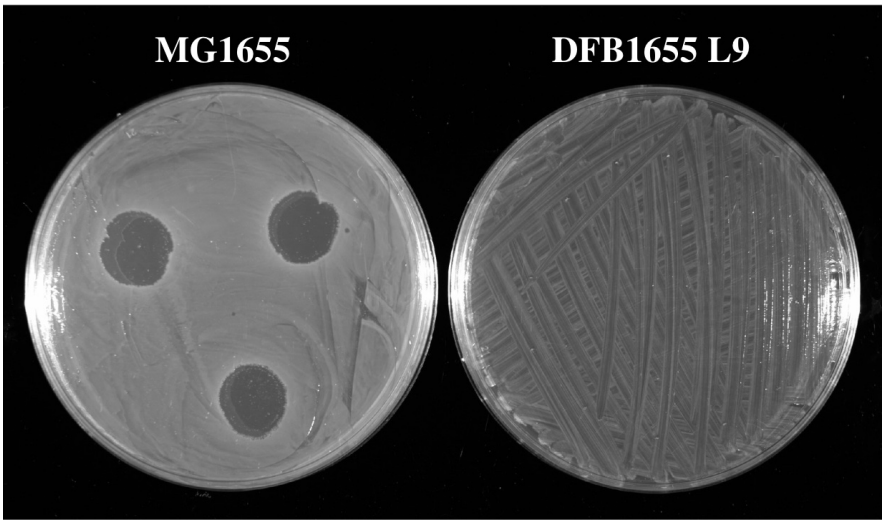

FIG. 2 MG1655 was susceptible to and DFB1655 L9 was resistant to T4 bacteriophage infection. Three 8 µL droplets of T4 bacteriophage were dispensed onto freshly swabbed MG1655 and DFB1655 L9 lawns, incubated for 16 hours at 37°C, then imaged using the ChemiDoc MP Imaging System (Bio-Rad).

T4 bacteriophage stock titer was 2.84 x 10^8 PFU/mL. To determine the concentration of T4 bacteriophage, a double agar overlay plaque assay was employed. Serial dilutions of bacteriophage stock were mixed with MG1655 and overlay agar, poured over underlay LB agar, then incubated overnight at 37°C. The number of plaques formed was counted on the following day, which was used to calculate the phage titre of 2.84 x 10^8 PFU/mL (Table S2).

O^{16}-antigen present in LPS extracted from DFB1655 L9, but not MG1655. MG1655 does not express O^{16}-antigen due to an insertion in the *wbbL* gene, whereas DFB1655 L9 expressed LPS with O^{16}-antigen (13). Due to the additional component of O^{16}-antigen in DFB1655 L9 LPS, it has a higher molecular weight than MG1655 LPS which has LPS alone (13). Browning *et al.* and Chiu *et al.* had previously characterized the LPS profile of MG1655 and DFB1655 L9 and observed a large smear around ~60 kDA only in DFB1655 L9 LPS extracts, representing O^{16}-antigen (6, 13). The presence or absence of O^{16}-antigen in the LPS extracts from MG1655 and DFB1655 L9 was confirmed by SDS-PAGE and subsequent silver staining (Fig. 3). A large smear between 60 and 100 kDA was observed in the DFB1655 L9 LPS profile but not in the MG1655 LPS profile (Fig. 3). This indicated that LPS with O^{16}-antigen was extracted from DFB1655 L9 and LPS without O^{16}-antigen was extracted from MG1655. These results were consistent with Browning *et al.* and Chiu *et al.*'s results (6, 13).

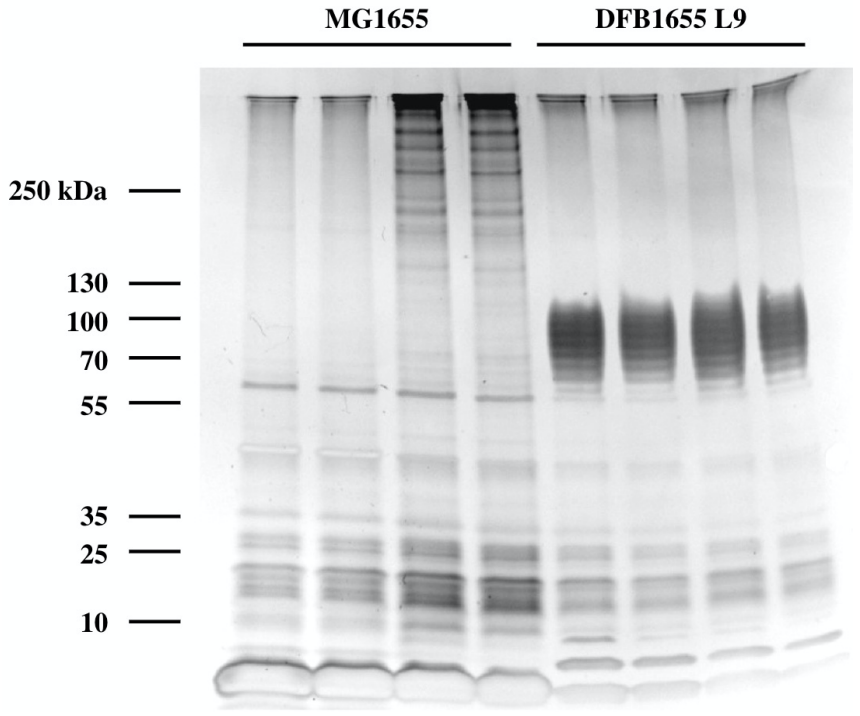

FIG. 3 LPS extracted from DFB1655 L9 contained O^{16}-antigen, while LPS extracted from MG1655 did not. LPS was extracted from MG1655 and DFB1655 L9, then subjected to SDS-PAGE and silver staining. Marker sizes (kDa) were superimposed from the PageRuler^{TM} PreStained Protein Ladder ran on a separate silver stained SDS-PAGE gel (Fig. S1). As indicated by the ~60-100 kDa smear, O^{16}-antigen was only observed in LPS extracted from DFB1655 L9.

Soluble O^{16}-antigen containing LPS from DFB1655 L9 delayed T4 bacteriophage adsorption to MG1655. To investigate the effects of soluble O^{16}-antigen on T4 viral adsorption, we performed a time series adsorption assay, which is adapted from Dimou *et al*. T4 bacteriophage was incubated for 2 hours with either LPS with or without O^{16}-antigen extracted from DFB1655 L9 or MG1655, respectively. The two treatment groups and a control group, which was T4 phage without LPS incubation, were then added to a liquid culture of log phase MG1655. The free phage concentration, representing the unadsorbed phage, was measured every minute until 10 minutes, as well as at 15 and 25 min.

A similar trend was observed between the control group and the treatment group in which T4 was incubated with LPS without O^{16}-antigen from MG1655. Free phage concentration decreased from 100% at t = 1 minute to 3.10% and 2.99% at t = 5 minutes, respectively (Fig. 4). However, a delayed decrease of free phage concentration was observed in the treatment group in which T4 was incubated with LPS with O^{16}-antigen from DFB1655 L9, with 17% of the initial free phage concentration still remaining at t = 5 minutes (Fig. 4). Beyond t = 5 minutes, the free phage percentage of the three groups gradually decreased, all reaching ~2% of initial free phage concentration at t = 10 minutes (Fig. 4). Given a multiplicity of infection (MOI) in the 10^{-3} range for all three groups, it can be safely assumed that available free phage have attached to MG1655 cells by t = 10 minutes (Table S3) (18). At t = 25 minutes, an increase of free phage in the supernatant was observed for the control group and the O^{16}-antigen LPS complex treated group. The phage concentration had considerably exceeded the original phage concentration at t = 1 minute, suggesting the T4-mediated cell lysis occurred between t = 15 minutes and t = 25 minutes (Table S3). Overall, these results show that T4 bacteriophage adsorption to MG1655 is delayed by incubation with soluble O^{16}-antigen LPS complex. The number of free T4 bacteriophages decreased more gradually when incubated with LPS extracted from DFB1655 L9 in comparison to incubation with LPS without O^{16}-antigen from MG1655 or without LPS incubation altogether.

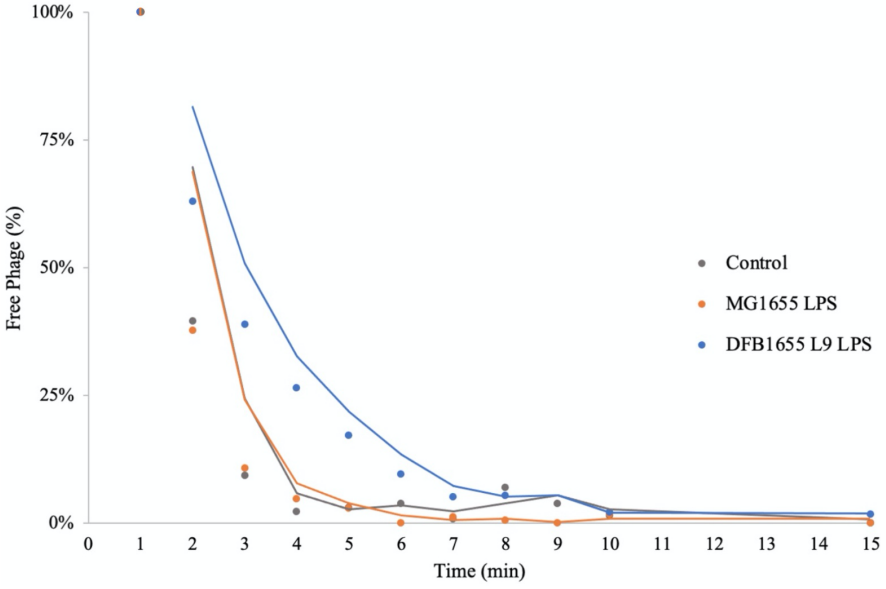

FIG. 4 Soluble O^{16}-antigen-LPS complex from DFB1655 L9 delayed T4 adsorption to MG1655. T4 bacteriophage was incubated for 2 hours with LPS with O^{16}-antigen from DFB1655 L9 (blue) or LPS without O^{16}-antigen from MG1655 (orange). These two treatment groups and a control, T4 bacteriophage without LPS incubation (gray), were subjected to a double agar overlay adsorption assay with MG1655. Free phage concentration is shown at 1-10 minutes and 15 minutes, with two period moving average trendlines for each group.

DISCUSSION

Previous studies have demonstrated that the presence of O^{16}-antigen in the LPS of *E. coli* K-12 confers resistance toward T4 phage infection, however the exact mechanism remains unknown (2, 5-12, 14). It has been demonstrated that O^{16}-antigen decreases infection at the adsorption stage (7, 9-11). Here, we provide further insight into the mechanism of O^{16}-antigen-mediated resistance of T4 phage in *E. coli* K-12, by testing the effect of purified O^{16}-antigen on phage adsorption.

As MG1655 and DFB1655 L9 are isogenic strains, observed differences in downstream experiments are attributable to isolated O^{16}-antigen. Therefore, additional bands aside from those indicating the presence or absence of O^{16}-antigen were assumed to be consistent across the strains. The presence of O^{16}-antigen in DFB1655 L9 derived samples and absence in

MG1655 derived samples were observed by silver-stained SDS-PAGE gel. These bands may be representative of the components within or associated with LPS, and may include additional molecules such as protein and/or carbohydrates that remain in the supernatant during extraction. Furthermore, the nature of the LPS extraction relies on the separation of LPS due to particle density in a final centrifugation step. Other particles not strictly O^{16}-antigen-containing LPS or other LPS with similar densities may also remain in the supernatant and are also carried forward in downstream applications. Replicate gels confirming O^{16}-antigen extraction consistently demonstrated successful isolation, however, variation in the banding pattern for bands that did not indicate the presence/absence of O^{16}-antigen were noted. These variations were attributed to differences in LPS resuspension methods prior to gel loading, as molecules, including LPS, may adhere to polymeric surfaces (19). For future applications with LPS, rigorous vortexing for a minimum of 2 to 3 minutes may aid with result consistency. Our approach to extract LPS was adapted from the manufacturer protocol, with a substitution of sonication with sand-aided mechanical lysis during the separation of LPS from the cell debris, which was found to stick to the pelleted sand. The potential presence of unwanted particles on the sand prior to autoclaving and use in LPS extraction was not tested, and may be a consideration in future applications. Although LPS could be extracted without the added mechanical lysis step, cell debris could not be pelleted. Thus, the inclusion of this mechanical lysis step likely reduced unwanted cell debris from being extracted with the LPS containing supernatant.

To elucidate the effects of soluble O^{16}-antigen on T4 adsorption to the surface of MG1655, free T4 phage, not bound to MG1655 was quantified through a time series adsorption assay. It was hypothesized that any interactions between O^{16}-antigen and T4 that affected T4 adsorption would be reflected in the concentration of free phage remaining in the supernatant. It was found that pre-treatment of T4 with O^{16}-antigen resulted in a delay in the adsorption of T4 onto the surface of MG1655. A higher concentration of free phage was measured in the supernatant of the experimental group compared to controls with LPS lacking O^{16}-antigen and LPS absent controls. Increases in free phage concentration in the supernatant after 25 minutes were attributed to the release of new T4 virions that had replicated inside the bacterial cells, hence this time point was not included in the analysis (Table S3). It was assumed that the 600-fold dilution of LPS treated T4 caused by addition to the overlay layer in the double agar overlay assay resulted in the dissociation between T4 and LPS. The eventual adsorption of O^{16}-antigen treated T4 onto the surface of MG1655 suggests that while O^{16}-antigen may play a role in hindering MG1655 infection by T4 by blunting the adsorption rate, it is not solely responsible for conferring T4 resistance in DFB1655 L9. Alternatively, it may suggest that the O^{16}-antigen to phage ratio was inadequate to confer resistance.

Several models of O^{16}-antigen mediated resistance have been proposed, including O^{16}-antigen preventing T4 from adsorbing to the bacterial surface, from binding to surface receptors such as OmpC, from replicating, or from releasing (7, 9-11, 14). Dimou *et al.* showed that in an adsorption assay, the concentration of free phage remained constant in DFB1655 L9 culture, which supports the model proposing that O^{16}-antigen prevents T4 from adsorbing to DFB1655 L9 (9). The delay of T4 adsorption when treated with O^{16}-antigen-containing LPS supports this model. This suggests that soluble O^{16}-antigen may be binding to phage in the extracellular environment and inhibiting its ability to adsorb to the cell surface. It is possible that experimental parameters such as the ratio of O^{16}-antigen to T4 or the incubation duration for treating phage with O^{16}-antigen may be insufficient to confer the same protective effects observed in previous studies. Not all phage particles may have been impacted by the presence of O^{16}-antigen LPS.

It is tempting to speculate that when T4 interacts with free LPS in the supernatant, the presence of O^{16}-antigen facilitates a similar reversible binding event involving long tail fibres that would normally occur at the cell surface. However, without other cell surface factors present, the phage cannot progress to the irreversible binding step involving short tail fibres. When used subsequently to inoculate susceptible cells, the T4 may dissociate from the free LPS complex, and adsorb to surface-bound O-antigen where it can then progress to the next stage of entry. This competitive step of reversible binding in the presence of free O-antigen may be responsible for the delay in adsorption. If binding between O-antigen and T4 is

irreversible, possible steric hindrance between the O-antigen T4 complex may decrease the ability of the phages from interacting with their receptors on MG1655.

Other mechanisms yet to be elucidated may also play a role in preventing T4 from establishing infection in DFB1655 L9. LPS is not the only component of the bacterial cell membrane that T4 interacts with and uses as a receptor (4). Literature to date has yet to validate the assumption that an insertion in *wbbL* solely affects O^{16}-antigen expression; it is possible that other variations between MG1655 and DFB1655 L9 strains exist and may be responsible for T4 resistance in DFB1655 L9. Furthermore, it is unknown whether O-antigen may have shielding effects that prevent T4 from effectively recognizing its receptors on the bacterial cell surface. Other molecules, such as surface proteins or glycans, potentially present on the surface of DFB1655 L9 bacterial cells, due to possible gene crosstalk during the restoration of *wbbL,* may be responsible for hindering adsorption. Additionally, the reverse is also possible whereby lack of these surface molecules could prevent adsorption if required to facilitate phage binding. Our findings that soluble O^{16}-antigen derived from DFB1655 L9 interacts with T4 phage in the extracellular environment to delay adsorption to MG1655 complements previous studies showing that surface-bound O^{16}-antigen confers complete resistance.

Limitations The ratios of T4 phage to LPS used in our adsorption assay were based on (v/v) ratios rather than molar ratios. The concentration of LPS stocks used in this study were approximated to be around 0.125 µg/mL based on the expected yield noted by the commercial LPS extraction kit used (Abcam), but were not quantified for exact concentration. Therefore, whether the molar concentration of LPS exceeded that of T4 virions in treatment conditions is unknown. If not exceeded, this could suggest that a greater concentration of O^{16}-antigen could lead to a greater delay in T4 adsorption or possible protection from infection. Hence, such effects cannot be presently concluded. When confirming the extraction of LPS, the silver staining technique did not allow for clean resolution of unstained protein ladders, which may be due to undesirable reactions between silver staining reagent and ladder solution. Therefore, pre-stained protein ladders were used instead for approximating the expected band location of O^{16}-antigen-containing LPS.

Additionally, the time series adsorption assay was based on the assumption that a 1:600 dilution of LPS-treated T4 phage to the MG1655 culture causes T4 bacteriophage and LPS to dissociate. This ratio was an arbitrary decision based on the protocol published by Dimou *et al.* (9), and future studies should validate whether this ratio is sufficient for complete phage-LPS dissociation. In addition, only two biological replicates for adsorption assay were conducted, therefore we were not able to test for statistical significance. Only the second biological replicate was employed for data analysis, and the first trial was excluded due to technical error.

Conclusion Previous studies have demonstrated that the presence of O^{16}-antigen in the LPS of *E. coli* K-12 confers resistance towards T4 infection by hindering phage adsorption to the cell surface. Here, we describe an effective approach using mechanical cell lysis and protein digestion to isolate LPS with and without O^{16}-antigen from DFB1655 L9 and MG1655 strains, respectively, in order to determine the effect of purified O^{16}-antigen on T4 adsorption to *E. coli* K-12. A time series adsorption assay from 1 minute to 25 minutes post inoculation showed that T4 phage treated with soluble O^{16}-antigen LPS derived from DFB1655 L9 remained in the culture supernatant longer than controls without O^{16}-antigen treatment, but eventually phage in the supernatant of all experimental groups depleted. Therefore, we conclude that pretreatment with O^{16}-antigen derived from DFB1655 L9 was able to delay viral adsorption to susceptible MG1655 cell surfaces, but did not confer long term resistance.

Future Directions The present study investigated the role of O^{16}-antigen in inhibiting T4 phage infection, however, different strains of *E. coli* express different serotypes of O-antigen (20). Future work could aim to investigate whether other serotypes also confer resistance to T4, and if they act in a similar mechanism. Given that the various serotypes of O-antigen differ with respect to their specific sugar composition, they may also interact differently with other bacteriophages, which should be further investigated.

To further elucidate the mechanism of resistance, it would also be a worthwhile avenue of investigation to see if the interaction between O-antigen and phage is reversible or irreversible. Possible approaches to this would be to perform adsorption assays over an increased time frame and include a shearing step to detach reversibly-bound phage, or visualizing involvement of phage long and short tail fibers through electron microscopy.

In addition, future replications of this study should adjust the LPS/O-antigen to phage ratio to provide a better understanding of the extent of resistance that may be conferred via this mechanism. For instance, the ratio used in this study was insufficient to confer resistance. A logical next step would be to quantify the extracted LPS then replicate this study with a range of LPS-phage ratios (i.e. an O-antigen concentration gradient) to allow for potential identification of a relationship between O-antigen quantity and degree of resistance, which can contribute meaningfully to our understanding of the mechanism.

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CONTRIBUTIONS

All authors contributed equally to protocol creation and manuscript writing and editing. The breakdown of the manuscript sections are as follows. WJ and XK covered the materials and methods and results, LM and JI oversaw the discussion and conclusion, and Jiang wrote the introduction and future directions. The abstract was a collective effort of LM, XK, and WJ. Experimental contributions are as follows. Authors JI and WJ lead efforts to validate the identity and functionality of all stock strains. Authors LM and XK lead efforts to extract LPS from bacterial strains and confirm extraction. All authors contributed to the execution and assessment of experiments evaluating soluble O^{16}-antigen on T4 infectivity.

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