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Escherichia coli O Antigen Serotype O16 Is a Restriction Factor for Bacteriophage T4 Infection

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The infection of *Escherichia coli* by bacteriophage T4 has long been investigated, providing key insights into viral life cycles, genetics, and phage therapy to combat bacterial disease. Alterations in the structure of the bacterial outer membrane, including LPS and OmpC, have been shown to play a key role in determining the tropism of T4. O antigen is present in the outer membrane of various bacterial species, including certain pathogenic serotypes of *E. coli*. While O antigen has been demonstrated to convey resistance to bacteriophage infection in other bacterial species, its role in T4 resistance in *E. coli* has not been explored. Here, we demonstrate a novel role for *E. coli* O antigen in restricting bacteriophage T4 infection using a laboratory-adapted *E. coli* strain with restored O antigen synthesis. Compared to the parental substrain, the O antigen-producing substrain resisted bacteriophage lysis across all multiplicities of infection tested, and showed comparable growth kinetics with or without T4 infection. In contrast, T4 infection exhibited robust bacterial lysis for the parental, non-O antigen-producing substrain. We demonstrated that this protective effect was not attributable to irreversible binding to secreted factors. This strongly supports the notion that the presence of O antigen alone can alter a susceptible strain of *E. coli* to become resistant to bacteriophage lysis, and underscores the relevance of external bacterial modifications in the development of phage resistance.

It has been well documented that numerous strains of *Escherichia coli* are susceptible to infection and lysis by bacteriophage T4 (1-3). Bacteriophage T4 is a large bacteriophage, consisting of a 169 kb genome packaged in an icosahedral head attached to a hollow tube, which is in turn attached to long tail fibers that mediate the initial binding of phage to host bacterial cells (4). The life cycle of T4 begins with infection of a susceptible bacterium, followed by hijacking of host machinery to rapidly produce many virions. Breakdown of the bacterial cell wall results in host cell death and lysis, thus releasing thousands of infectious bacteriophage progeny into the environment (4). Like many bacteriophages, T4 viral binding and entry is dependent on direct binding of its tail fibers to exposed bacterial residues (1). In some bacterial strains, such as *E. coli* B, T4 adsorbs to a glucose residue on the exposed end of lipopolysaccharide (LPS) found on the cell surface (1). In other strains, such as *E. coli* K-12, T4 adherence requires binding to the outer membrane protein OmpC as well as the core-lipid A region of LPS (2). Cells that lack both the OmpC protein and the outer glucose region of LPS are extremely resistant to T4, though removal of OmpC when LPS was modified to expose a distal glucose residue was not observed to affect infectivity, highlighting the multiple parallel structural modifications that impact bacteriophage infectivity (2). Furthermore, the same study highlighted multiple mechanisms by which bacterial modifications may restrict bacteriophage infection and lysis, including steric hindrance, inactivation of phage via cell envelope components, and inactivation of phage via secreted soluble factors (2). Thus, *E. coli* bacteria have evolved an array of mechanisms that convey resistance to T4 infection, primarily via the modification of host receptors.

E. coli strains are serotyped based on the presence of polysaccharide antigens in their cell membranes, including O antigen (3). While O antigen is not inherently pathogenic, many of the most pathogenic serotypes of *E. coli*, including O157:H7 and O104:H4, are marked by the presence of O antigen (5). O antigen is a structurally diverse polysaccharide component of LPS present on many strains of *E. coli*, and has been suggested to function as an inhibitor of T4 adsorption (1). Wild-type *E. coli* K-12 does not synthesize O antigen due to mutations in its *rfb* gene

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cluster, which contains genes involved in O antigen production. One such gene is *wbbL*, which encodes a rhamnose transferase involved in O antigen synthesis. In most substrains of *E. coli* K-12, *wbbL* is inactivated by the presence of a 1,195 base pair IS5 insertion element (6-9).

A substrain of *E. coli* K-12 was previously engineered to synthesize O antigen by chromosomal insertion of regions of an intact *rfb* gene locus (8). O antigen synthesis in *E. coli* K-12 substrain MG1655 has more recently been restored by chromosomal insertion of an intact, uninterrupted *wbbL* gene from *E. coli* strain WG1 (9). This newly modified substrain, DFB1655 L9, synthesizes the O antigen serotype O16, and was found to have marked phenotypic differences from wild-type *E. coli* K-12 (9). In contrast to wild-type MG1655, DFB1655 L9 was pathogenic to *Caenorhabditis elegans*, as it was protected from the effects of mechanical shearing, less able to produce biofilms, and more resistant to both bacteriophage P1 and plasmid transformation (9). O antigen production in DFB1655 L9 did not appear to affect growth, cell viability, or alter outer membrane composition, including the production of OmpC (9).

The presence of O antigen and modifications to its structure have been shown to act as defense mechanisms against phage infection in *Salmonella enterica* serovar Typhimirium (10). Additionally, production of O antigen has been demonstrated to protect both *Salmonella montevideo* and *E. coli* K-12 derivatives from complement-mediated bacterial lysis (11, 12). However, the effects of O antigen on infectivity by bacteriophage T4 in *E. coli* have not been specifically characterized. Using *E. coli* K-12 as a model system to study this interaction is desirable, since this strain is nonpathogenic to humans and is used widely in molecular biology research (9, 10). Here, we describe for the first time the role of O antigen synthesis by *E. coli* MG1655 in restricting infection and lysis by bacteriophage T4. We hypothesized that synthesis of O antigen by E. coli K-12 results in greater resistance to infection and lysis by bacteriophage T4.

MATERIALS AND METHODS

Bacterial strains used. *E. coli* K-12 substrain MG1655 and *E. coli* K-12 substrain DFB1655 L9 were obtained from the Henderson laboratory at the University of Birmingham, courtesy of Douglas F. Browning. MG1655 is a substrain unable to synthesize O antigen. DFB1655 L9, derived from MG1655, has restored ability to express O antigen serotype O16 through the chromosomal insertion of the *wbbL* gene (Table S1) (9). Bacteriophage T4 was obtained from Carolina Biological Supply (cat no. 12-4330).

Generation of primary phage lysates. An overnight culture of MG1655 in 5 mL of Luria_Bertani (LB) broth was diluted 1/5 and 1/50 in 5 mL amounts of LB Broth. 10 µL of bacteriophage T4 was added. Cultures with phage were grown overnight at 37°C, 200 rpm. 300 µL of chloroform was added to cultures, shaken vigorously, and left at 4°C overnight to settle. The upper aqueous fraction containing T4 was removed, filtered using a 0.22 µm filter, and stored at 4°C.

TABLE 1 Primers designed for amplifying fragments of T4 major capsid gp23, T7 capsid gp10a, and ΦX174 F capsid protein. The letters F and R denote the forward and reverse primers, respectively.

Gene	Sequence (5′ - 3′)	Size (bp)
T4 gp23	F: GCCATTACTGGAAGGTGAAGG	398
	R: TTGGGTGGAATGCTTCTTTAG	
T7 gp10a	F: CGAGGGCTTAGGTACTGC	295
	R: GGTGAGGTGCGGAACTTC	
ΦX174 F	F: GTACGCTGGACTTTGTGGGA	719
	R: TCGGGAGAGGAGTGGCATTA	

PCR amplification of phage genes to determine purity of phage extraction. PCR amplification of bacteriophage T4 lysates using T4, T7, and ΦX174-specific primers (Table 1) was performed using Platinum® *Taq* DNA Polymerase (Invitrogen) kit components as per the manufacturer's instructions. Primers were ordered from Integrated DNA Technologies (IDT) and were used at final concentrations of 0.1 µM per reaction sample. The Bio-Rad T100™ Thermal Cycler was programmed for a 10-minute initial denaturation step at 95°C, followed by 30 cycles of denaturation for 30 seconds at 95°C. The 30-second annealing phase of each cycle was run at 51.3°C and 52.9°C for all three primer sets, having been previously optimized by gradient PCR. Extension phase for each of the cycles was 1 minute at 75°C. All PCR products were separated on a 1.2% agarose gel in 1x TAE buffer at 100 V for 30 minutes and at 110 V for 30 minutes. Bands were visualized using SYBR™ Safe DNA Gel Stain (Invitrogen).

Isolation of *E. coli* K-12 substrains MG1655 and DFB1655 L9. Both *E. coli* substrains were streaked onto pre-warmed LB agar (1.5%) plates from LB agar stabs to obtain isolated colonies. MG1655 was streaked onto plain LB plates without added antibiotics. DFB1655 L9 was plated on LB supplemented with 50 µg/mL Kanamycin salt (Invitrogen). Plates were incubated overnight at 37°C.

Comparison of MG1655 and DFB1655 L9 growth rates by growth curve. Overnight cultures of MG1655 and DFB1655 L9 in 5 mL of LB broth were used to create 1/100 dilutions to a total volume of 25 mL (2 flasks per strain) or 50 mL (1 flask per strain). Flasks were incubated at 37°C, 200 rpm. Optical density at 600 nm (OD_{600}) was measured as a readout for cell turbidity at regular time intervals using a Pharmacia Biotech Ultrospec 3000.

SDS-PAGE of total cell lysates of MG1655 and DFB1655 L9 to compare protein content. Overnight cultures (0.1 mL) of MG1655 and DFB1655 L9 in LB broth were pelleted by centrifugation for 10 minutes at 10,600x *g* in an Eppendorf 5424 R tabletop centrifuge. Supernatants were discarded and pellets were resuspended in 120 µL of 1x sodium dodecyl sulfate (SDS) sample buffer, prepared as specified by Davis *et al.* (13). Samples were boiled at 95°C for 10 minutes and frozen at -20°C for storage. Prior to separation by SDS-PAGE, lysates were thawed and heated at 95°C for 1 minute. A 10-well NuPAGE™ Novex™ 4-12% Bis-Tris Protein Gel, 1.0 mm (Invitrogen) was placed in a Novex Mini-Cell

and filled with 1x NuPAGE™ MES SDS Running Buffer (Invitrogen). Varying amounts of lysate and 10 µL of Novex Sharp Pre-stained Protein Standard (LifeTech) were loaded into each well. Gel was run at 200 V for 5 minutes, then at 120 V for 85 minutes. Gel was rinsed with distilled water, incubated with 30 mL of InstantBlue Protein Stain (Expedeon) for 45 mins at 100 rpm at room temperature, and visualized immediately using a phone camera.

Colony PCR to detect *wbbL* in MG1655 and DFB1655 L9. PCR amplification of isolated colonies with *wbbL*-specific primers was performed using Platinum® *Taq* DNA Polymerase (Invitrogen) kit components as per the manufacturer's instructions. Primer sequences were sourced from Browning *et al*. (9) (Table 2). PCR template was generated by transferring a 10 µL pipette tip to isolated colonies of MG1655 and DFB1655 L9 and scraping the bottom of pre-labeled PCR tubes. Primers were ordered from Integrated DNA Technologies (IDT) and were used at final concentrations of 0.1 µM per reaction sample. The Bio-Rad T100™ Thermal Cycler was programmed for a 5-minute initial denaturation step at 95°C, followed by 30 cycles of denaturation for 30 seconds at 95°C. The 45-second annealing phase of each cycle was run at a gradient from 45.0°C to 55.0°C for both substrains. Extension phase for each of the cycles was 2.5 minutes at 75°C. All PCR products were run on a 0.8% agarose gel in 1x TAE buffer at 120 V for 10 minutes and at 80 V for 35 minutes. Bands were visualized using SYBR™ Safe DNA Gel Stain (Invitrogen).

LPS extraction and SDS-PAGE to detect O antigen production in MG1655 and DFB1655 L9. Overnight cultures of MG1655 and DFB1655 L9 were diluted to an OD_{600} of 0.5 and pelleted by centrifugation for 10 minutes at 10,600x *g* in an Eppendorf 5424 R tabletop centrifuge. Supernatants were discarded and pellets were resuspended in 200 µL of 1x SDS sample buffer, prepared as specified by Davis *et al* (13). Samples were boiled at 95°C for 15 minutes and cooled at room temperature for 15 minutes. 10 µL of proteinase K (10 mg/mL) was added to each lysate, and samples were incubated at 59°C for 3 hours. Samples were stored at -20°C. Prior to running on SDS-PAGE, lysates were thawed and heated at 95°C for 1 minute. A 10-well NuPAGE™ Novex™ 4-12% Bis-Tris Protein Gel, 1.0 mm (Invitrogen) was placed in a Novex Mini-Cell and filled with 1x NuPAGE™ MES SDS Running Buffer (Invitrogen). Varying amounts of lysate and 10 µL of Novex Sharp Pre-stained Protein Standard (LifeTech) were loaded into each well. Gel was run at 200 V for 60 minutes, rinsed with distilled water, stained with a SilverQuest (Invitrogen) silver staining kit according to manufacturer's instructions, and visualized immediately using a phone camera.

TABLE 2 Primers designed for amplifying the *wbbL* gene in *E. coli* K-12 strain MG1655. The letters F and R denote the forward and reverse primers, respectively.

Gene	Sequence (5′ - 3′)	Size (bp)
wbbL	F: CCCGAATTCATATGGTATATATAATAATCGTTTCCC	1994 (MG1655)
	R: CCCAAGCTTCTCGAGTTACGGGTGAAAAACTGATGAAATTC	799 (DFB1655 L9)

Double agar overlay plaque assay to assess differential infection of MG1655 and DFB1655 L9 by bacteriophage T4. Reagents were prepared according to the standard protocol by Kropinski *et al*. (14). Bacteriophage T4 was serially diluted in LB broth (1 mM $CaCl_2$). 100 µL of overnight cultures and 100 µL of T4 dilutions were added to 3 mL tubes of 0.7% LB agar (1 mM $CaCl_2$), mixed, and poured onto 1.5% LB agar plates (1 mM $CaCl_2$). No T4 was added to control plates. After solidifying, plates were incubated overnight at 37°C. Plaques were counted the following day. Titer of bacteriophage T4 was calculated by averaging the number of plaques from countable plates, yielding a value of 2.28 x 10^{10} plaque forming units (pfu)/mL. Pfu, a measurement of the number of particles capable of forming viral plaques, is used to quantify the number of infectious virions per unit volume, and is thus used as a surrogate for infectious and lytic capability.

Growth curve with phage inoculation to assess differential infection of MG1655 and DFB1655 L9 by bacteriophage T4. Overnight cultures of MG1655 and DFB1655 L9 in 5 mL of LB broth were used to create 1/100 dilutions to a total volume of 50 mL each in LB Broth supplemented with 1 mM $CaCl_2$. Flasks were incubated at 37°C, 200 rpm until reaching an OD_{600} of at least 0.1. 40 µL of T4 was added to treatment flasks, reaching a multiplicity of infection (MOI) of 0.13 to 0.21 per flask. MOIs used were consistent with concentrations used for T4 infection in the literature (3). No T4 was added to control flasks. Cultures were incubated at 37°C, 180 rpm and OD_{600} was measured at regular time intervals using a Pharmacia Biotech Ultrospec 3000.

Incubation of T4 with *E. coli* soluble factors to determine mechanism of inhibition by DFB1655 L9. Overnight cultures of MG1655 and DFB1655 L9 were centrifuged for 10 minutes at 10,600x *g*. 4.5 mL of both supernatants as well as plain LB (1 mM $CaCl_2$) were filtered with a 0.45 µm syringe filter into three sterile tubes. 0.5 mL of purified T4 lysate was added to each of the three tubes, incubated for 2 hours at 37°C, 180 rpm and stored overnight at 4°C. The following day, 6 mL LB (1 mM $CaCl_2$) was aliquoted into sterile tubes and mixed with 200 µL of an overnight culture of MG1655. 200 µL of each T4 incubation, as well as 200 µL of freshly diluted T4 (1/10) was added to tubes containing MG1655, reaching an MOI of roughly 1.0. One set of tubes contained no phage as a negative control. Tubes were incubated at 37°C, 180 rpm and OD_{600} was measured at regular intervals with a Pharmacia Biotech Ultrospec 3000.

Statistics. All statistical analysis was performed on GraphPad Prism v7.02 (GraphPad Software, La Jolla, CA). All pairwise comparisons were assessed by unpaired Student's t-test. Data are presented as mean ± SD. $P \leq 0.05$ was considered to reflect statistically significant differences between compared groups. The data shown are representative of three or more replicates and all samples in a group are biological replicates.

RESULTS

Growth kinetics and total protein production in MG1655 and DFB1655 L9. Both substrains used in this experiment displayed no discernable difference in growth over time. When incubated in liquid culture, both substrains entered exponential growth and stationary phase at approximately the same time post-inoculation (Fig. 1A). Additionally, comparable protein content was observed between both

substrains upon SDS-PAGE of total cell lysates. In DFB1655 L9, there was a ~48 kDa band that was only faintly present in MG1655 (Fig. 1B). These findings were consistent with those described by Browning *et al.* (9).

O antigen production upon reintroduction of the *wbbL* gene locus. To confirm the presence of a functional 799 bp *wbbL* gene in DFB1655 L9 and the absence of said gene in MG1655, colony PCR was performed on both substrains using primers flanking the *wbbL* gene. In MG1655, PCR resulted in amplification of a single 2 kb product while in DFB1655 L9, two products were amplified: one at 800 bp and another at 2 kb (Fig. 2A). Upon confirmation of the presence of an 800 bp *wbbL* gene in DFB1655 L9, extraction of LPS was conducted to confirm the presence of O antigen synthesis in this substrain. LPS extracts for both substrains were separated by SDS-PAGE, and the LPS extracted from DFB1655 L9 was found to contain a large smear between 50 and 60 kDa, consistent with the known size of O antigen (Fig. 2b). This was not observed in extracted MG1655 LPS, suggesting the absence of O antigen synthesis in this substrain. The presence of O antigen in DFB1655 L9 and not MG1655 was further validated via aqueous phenol LPS extraction and subsequent silver staining of both

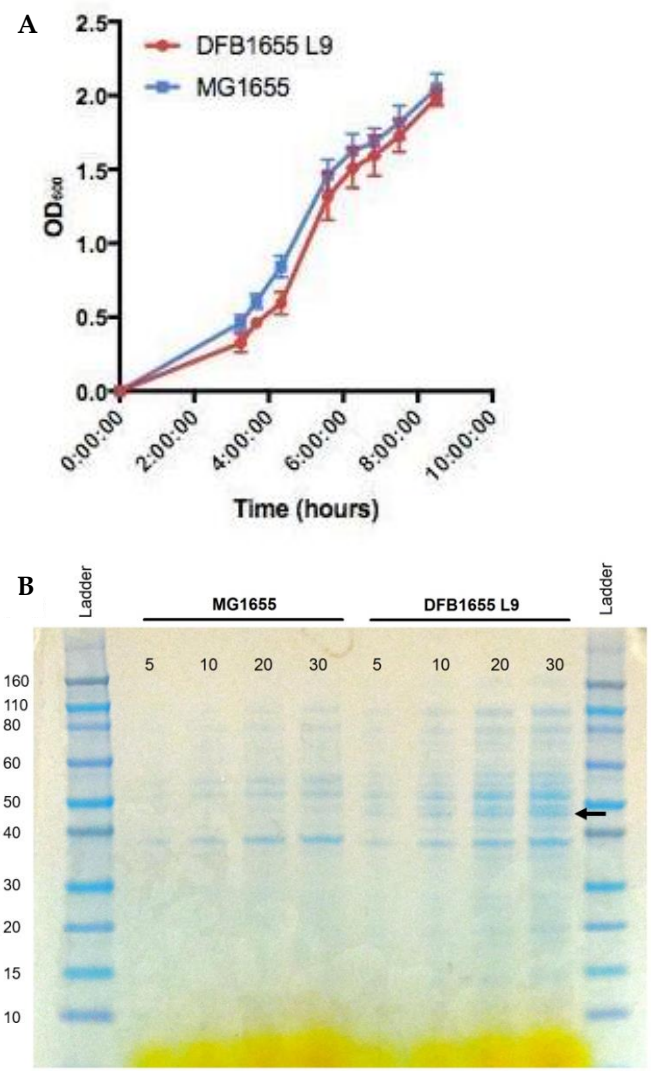

FIG. 1 *Escherichia coli* K-12 strain DFB1655 L9 displays comparable growth kinetics and total protein production as parental MG1655. (A) Single colonies of MG1655 and DFB1655 L9 were grown in LB broth without antibiotics at 37°C and OD_{600} measured at denoted time points. All experiments were performed in triplicate. No discernible difference was observed in growth kinetics between the two strains. **(B)** Total bacterial lysates obtained from MG1655 and DFB1655 L9 were analyzed via SDS-PAGE and subsequent Coomassie staining as described in Materials and Methods. A range of lysate concentrations, as denoted, were used per substrain. No apparent differences between total protein content, apart from a ~48 kDa band observed in DFB1655 L9 denoted by the arrow, were observed. Numbers indicate volume of lysate loaded. Representative images of 3 experiments are shown.

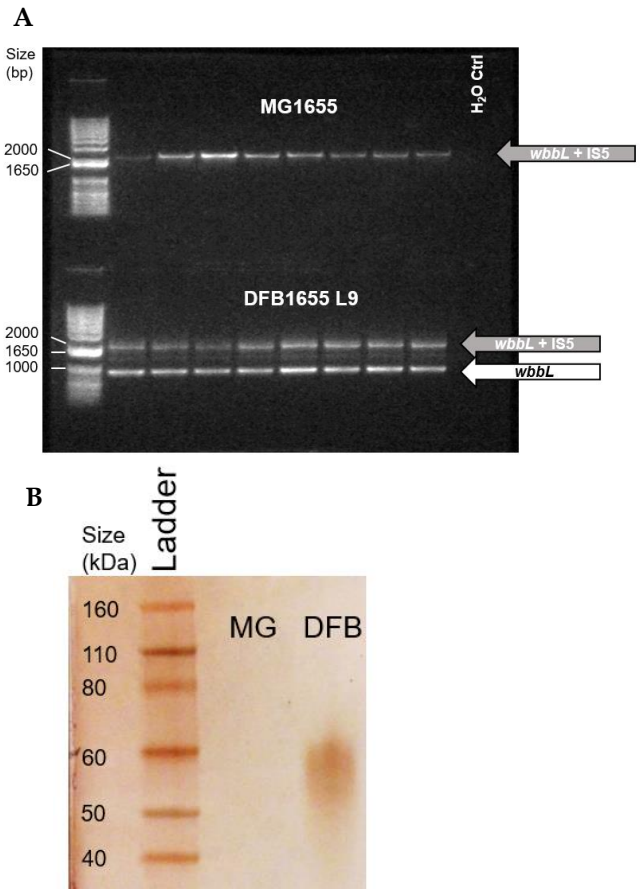

FIG. 2 *Escherichia coli* K-12 strain DFB1655 L9 produces O antigen upon reintroduction of the *wbbL* locus. (A) Colony gradient PCRs of MG1655 and DFB1655 L9 were performed. The ~800 bp amplicon (denoted by the white arrow) representing the *wbbL* gene is seen in DFB1655 L9 only, whereas the ~2 kbp amplicon (denoted in grey) representing the *wbbL* gene with the 1.2 kb IS5 insert is observed in both strains. H_2O PCR negative control run on top right of gel. **(B)** LPS extraction via Proteinase K of MG1655 and DFB1655 L9 and subsequent SDS-PAGE and silver staining were performed as described in Materials and Methods. O antigen, as visualized by the ~50-60 kDa smear, is observed in the DFB1655 L9 LPS extract only.

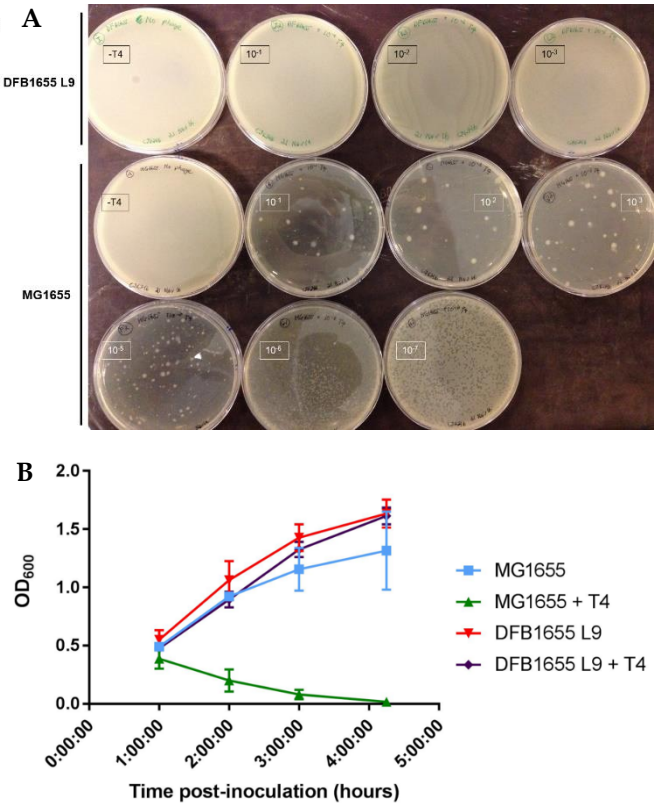

FIG. 3 Production of O antigen is sufficient to restrict bacteriophage T4-mediated lysis of *Escherichia coli* K-12 substrain MG1655. (A) Double overlay plaque assays were performed as described in Materials and Methods with T4 and MG1655 or DFB1655 L9 at the MOIs indicated. Following overnight incubation, no plaques were visible in the DFB1655 L9 substrain at any MOI. Representative images are shown. Numbers indicate serial dilutions of T4 from a stock containing 2.28 x 10^{10} pfu/mL. Representative images of 3 experiments are shown. **(B)** Liquid cultures of MG1655 and DFB1655 L9 were inoculated with bacteriophage T4, as denoted. OD_{600} readings were taken at the timepoints given post-inoculation. T4-inoculated MG1655 demonstrated a marked decline in bacterial concentration, which was not observed for inoculated DFB1655 L9 or either of the uninoculated strains.

substrains (Fig. S1). These results were consistent with those presented by Browning *et al.* (9).

DFB1655 L9 is resistant to bacteriophage T4-mediated lysis. Upon confirmation of the presence of O antigen in DFB1655 L9 and the absence of O antigen in MG1655, a plaque assay was conducted to determine whether T4 was able to infect either substrain. The purity of bacteriophage T4 stock used was first validated through PCR (Fig. S2). T4 formed distinct plaques in MG1655; the concentration of T4 used in these assays was determined to be 2.28 x 10^{10} pfu/mL. When incubated with DFB1655 L9, no plaques were visible at any plated concentration of T4 (Fig. 3A). Liquid cultures of MG1655 and DFB1655 L9 were subsequently grown and inoculated with T4 during early exponential phase at an MOI between 0.1 and 0.2 to confirm resistance to phage infection. MG1655 inoculated with T4 showed nearly complete clearing and lysis after four hours of growth, while DFB1655 L9 inoculated with T4 showed no discernible difference from cultures not inoculated with phage ($p < 0.0001$) (Fig. 3B). Interestingly, prolonged incubation of MG1655 with T4 resulted in detectable levels of bacteria 12 hours post-inoculation (Fig. S3).

Soluble factors of *E. coli* K-12 do not inactivate bacteriophage T4. To investigate the mechanism by which O antigen may inhibit T4 binding and lysis, we sought to determine whether soluble factors produced by DFB1655 L9 inhibited T4 infection by chemical inactivation. T4 was incubated in the supernatants of overnight cultures of DFB1655 L9 and MG1655. After overnight incubation at 4°C to ensure adequate exposure time to soluble factors, T4 was used to infect liquid cultures of MG1655 at an MOI of 1.0. T4 incubated with soluble factors from either substrain showed no detectable difference in its ability to lyse MG1655 compared to pure T4 (all pairwise comparisons $p > 0.05$) (Fig. 4).

DISCUSSION

Many of the most pathogenic serotypes of *Escherichia coli*, including O157:H7 and O104:H4, are marked by the presence of O antigen in their cell membranes (5). Thus, there has been significant interest in potential mechanistic roles of O antigen in toxicity, evasion of immune responses, and resistance to treatment. Here, we describe a novel role for *E. coli* O antigen in restricting bacteriophage infection. Using a substrain derived from *E. coli* K-12, substrain MG1655, we

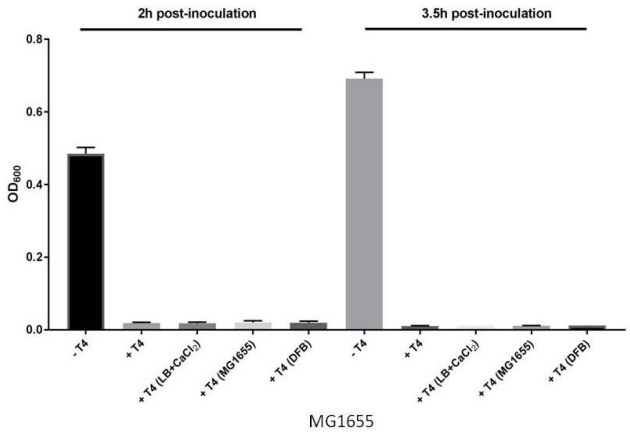

FIG. 4 Soluble factors of *Escherichia coli* K-12 do not inactivate bacteriophage T4. *E. coli* substrain MG1655 was left uninoculated (- T4) or inoculated with propagated T4 (+ T4), T4 incubated with 1mM $CaCl_2$-supplemented LB (LB+$CaCl_2$), T4 incubated with MG1655 supernatant (MG1655), or T4 incubated with DFB1655 L9 supernatant (DFB). All cultures were subsequently incubated and OD_{600} readings taken at the timepoints denoted. No differences were observed between lytic ability of any of the incubated and unincubated T4 bacteriophages tested.

demonstrate that the rescue of O antigen synthesis alone is sufficient to reduce bacterial lysis upon exposure to bacteriophage T4. This is consistent with previous reports which implicate higher percentages of LPS bearing O antigen in the evasion of phage and complement-mediated bacterial lysis in *Salmonella* and *Escherichia* species (10-12).

Providing support for the results described by *Browning et al.*, we have demonstrated that the restoration of an uninterrupted *wbbL* locus is sufficient to rescue O antigen synthesis in *E. coli* MG1655 (9). This is also consistent with the results observed by *Hong et al.*, who used a trans complementation in lieu of a chromosomal insertion (19).

Importantly, DFB1655 L9 did not display any major differences from the parental MG1655 substrain, both in terms of growth kinetics (Fig. 1A) and total protein production (Fig. 1B). A darker 48 kDa band was observed in DFB1655 L9 but not in MG1655 (Fig. 1B); this band was also observed in the data presented by Browning *et al.* (9). The WbbL rhamnosyltransferase produced by the *wbbL* gene is 31 kDa; the observed band may represent a larger, unprocessed version of the protein, and should be investigated in the future using mass spectrometry in order to elucidate whether or not this protein may have additional effects differentiating the DFB1655 L9 strain from MG1655. However, it is important to note that Browning *et al.* analyzed the two substrains via microarray and found no major differences related to metabolic activity (9). This lack of significant metabolic differences results in these two matched substrains presenting a tractable model with which to investigate the role of O antigen in isolation.

Herein we demonstrate that while T4 exerts a lytic effect on MG1655, DFB1655 L9 is completely resistant to T4-mediated bacterial lysis. As anticipated, inoculation with T4 resulted in extensive bacterial lysis across a range of MOIs, as visualized by the clearings in the double overlay plaque assays (Fig. 3A). In contrast, T4 did not form any visible plaques in DFB1655 L9 at any MOI tested (Fig. 3A). The kinetics of T4 lytic infection were further investigated through a growth curve and were consistent with the results of the plaque assays; no difference was observed between DFB1655 L9 whether in the presence of phage or not while T4 demonstrates robust lytic activity towards MG1655 (Fig. 3B). The similarity between DFB1655 L9 and MG1655 (Fig. 1) strongly suggest that the presence of O antigen alone is sufficient to restrict infection by bacteriophage T4. Strikingly, we observed that MG1655 growth was restored at later timepoints post-inoculation (Fig. S3). This incomplete bacterial lysis suggests the natural development of phage resistance, and such alternative resistance mechanisms should be explored further.

To explore the mechanisms underlying O antigen-mediated phage resistance, we investigated the effects of *E. coli* soluble factors on T4 infection. T4 was incubated with supernatant from either MG1655 or DFB1655 L9 prior to inoculation with MG1655 culture. No differences in bacterial lysis were observed (Fig. 4), suggesting that DFB1655 L9 does not exert its restriction on T4 phage infection via irreversible binding to secreted factors. O antigen is highly diverse structurally, and consists of repeating oligosaccharide subunits up to 40 repeat units long (20). The presence of O antigen has been shown to decrease binding of both host antibodies and complement in *Salmonella typhimurium* and *E. coli* by sterically blocking access to the bacterial surface (20). Given the presence of O antigen on the exterior surface of LPS and therefore the bacterium, O antigen may be able to inhibit T4 adsorption via steric hindrance. Given that T4 infection requires an initial binding step, with its long tail fibers binding the bacterial surface, O antigen may be able to hinder phage infection in a similar manner to the aforementioned inhibition of both antibodies and complement.

We describe for the first time a role for *E. coli* O antigen in restricting bacteriophage T4 infection. Using an O antigen-producing substrain derived from *E. coli* K-12 MG1655, we validated the synthesis of O antigen via restoration and expression of the *wbbL* gene. Importantly, we confirmed that the *wbbL* gene insert does not noticeably affect growth rate or total protein production. Production of O antigen is sufficient to restrict T4-mediated bacterial lysis at across a range of multiplicities of infection and timepoints, as validated experimentally both using a double agar overlay plaque assay and a liquid culture growth curve. Preliminary experiments into the mechanisms of O antigen-mediated resistance suggest that this resistance not due to irreversible binding to soluble factors. This project will help build a foundation for studying the effects of different O antigen serotypes on the infectivity of a wide variety of bacteriophages. This may also provide insight into the use of bacteriophage T4 as a therapeutic antimicrobial agent against enteropathogenic *E. coli*, as efficacies of such therapies have historically been mixed (15, 16).

FUTURE DIRECTIONS

To further investigate the role O antigen plays in resistance to infection with bacteriophage T4, repetition of this experiment using different types of O antigen would be

useful. The type of O antigen used in this experiment was O16, however O antigen varies widely between different serotypes of *E. coli*; for example, the B strain of *E. coli* would produce O7 if the activity of its *wbbD* gene were restored (17). Alternatively, knocking out or knocking in the ability to synthesize a serotype of O antigen associated with pathogenic *E. coli* would provide useful insight into the relevance of these results to phage therapy. It would also be useful to establish the exact mechanism of resistance to phage conferred by O antigen. Additionally, there are a number of bacteriophages which could also be investigated using this strain, such as bacteriophage T7. T7 infects rough *E. coli*, but there is also some evidence that related strains are also able to infect smooth strains of *E. coli*, including the pathogenic strain *E. coli* O157:H7 (18).

Another potential avenue of investigation is the naturally occurring restoration of MG1655 growth observed after 12 hours of growth in the presence of T4. This observation suggests that selection for resistant strains of MG1655 occurs after long exposure to bacteriophage T4. Characterizing the differences which lead to this naturally occurring resistance could further improve our understanding of the T4 infection cycle and the natural co-evolution of phage and bacteria.

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