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Roles of Lipopolysaccharide and the Outer Membrane Protein OmpC on Bacteriophage T4 Infectivity in *Escherichia coli*

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Although previous studies have demonstrated that lipopolysaccharide (LPS) and the outer membrane protein OmpC are required for bacteriophage T4 infectivity in *Escherichia coli*, it is unclear whether they played a role in binding or in the downstream viral processes that followed. Such processes may vary from phage DNA injection to viral gene expression, assembly, or release. To investigate this further, phage binding and overlay plaque assays were performed on *E. coli* strains C149 (wildtype), C845 (LPS mutant), C842 (LPS mutant), and C157 (OmpC mutant). In this study, we report that LPS and OmpC play an essential role in one or more viral processes that follow binding. In addition, it was discovered that LPS and OmpC mutants C842 and C157, respectively, did not alter phage binding affinity but decreased phage binding stability. Moreover, the LPS mutant, C845, increased binding affinity to T4 phage but decreased binding stability. All these results suggest that modifications to LPS can influence both the affinities and stabilities of phage binding.

The T4 bacteriophage, a member of the T-even phage group, is an efficient infectious agent of *Escherichia coli* (*E. coli*) (4). Its structure consists of a DNA-containing head, a tail, as well as a baseplate which is attached to both short and long tail fibres (12). T4 phage employ a very complex entry mechanism, involving an ATP-dependent DNA injection process (7). Bacteriophages remain attached to the outer cell surface during infection and have evolved a mechanism to use their tail fibers for host cell recognition, attachment, and genome delivery (7).

T4 phage contain six tail fibers which are symmetrically organized around a hexagonal baseplate (1,3). Initial attachment to a host cell receptor is reversible, weak, and dependent upon the interaction between at least three long tail fibers and a glucose residue on of the outer core of the lipopolysaccharide (LPS) of the host cell surface (1). Previous studies have shown that these binding interactions are weak and may vary from hydrogen bonding to hydrophobic and electrostatic interactions (4). After the tail fiber binding has been consolidated, the baseplate then comes in contact with the host cell surface, causing the short tail fibers to eject and bind irreversibly to the heptose section of the inner core of the LPS (1). This transition from reversible to irreversible binding involves a number of host and phage components (3,7). After this conformational change, the tail sheath contracts, causing the tail tube to be forced through the outer cell membrane of the host (1). The tail tube then reaches the inner membrane, aided by the tail lysozyme, glycoprotein 5. The phage genome is then injected into the host cytoplasm (1). It is important to note that the irreversibly bound state can exist in two phases, one where the phage DNA has not been internalized and one where it has. Following DNA injection, the host cell transcription and translation machinery is used to produce phage specific early mRNA and proteins (1). Eventually, late mRNA and proteins are produced (1). These proteins include phage structural proteins as well as proteins involved in cellular lysis (1). Ultimately, infectious phage particles are assembled and the host cell undergoes lysis and releases phage progeny (11).

Previous studies have demonstrated that LPS and the outer membrane protein OmpC are required for bacteriophage T4 infectivity in *E. coli* (9,11,12). LPS is located exclusively in the outer leaflet of the outer membrane of gram negative bacteria (11). LPS exhibits features of both lipids and polysaccharides and its structure is divided into three regions: Lipid A, R-core, and O-polysaccharide (11). OmpC is an outer membrane protein that is involved in regulating the response of *E. coli* to the osmolarity of its environment (2). It is highly expressed during conditions of high osmolarity and repressed during conditions of low osmolarity (2). Particular studies found that *E. coli* K-12 strains that contained a mutation in their OmpC or LPS structures were resistant to T4 phage infection (9,12). Although these studies have helped identify that both LPS and OmpC are required for infectivity, it was unclear whether these structures played a role in binding or in downstream viral processes that followed. Such processes may vary from phage DNA injection to viral gene expression, assembly, or even release.

The purpose of this experiment was to determine whether LPS and OmpC are involved in the initial binding of phage or a viral process that followed

binding. An adjunct experimental goal was to determine if the mutations in LPS and OmpC influenced the affinities and stabilities of phage binding to *E. coli*. We achieved our goals by performing a phage binding and overlay plaque assay for *E. coli* strains C149 (wildtype), C845 (LPS mutant), C842 (LPS mutant), and C157 (OmpC mutant).

MATERIALS AND METHODS

Bacterial and Phage Strains. All *E. coli* strains C149 (wildtype), C845 (LPS mutant), C842 (LPS mutant), and C157 (OmpC mutant) were provided by Jennifer Sibley (Department of Microbiology and Immunology, University of British Columbia (UBC)). All strains have been described previously (9). T4 phage was provided by Dr. William Ramey (Department of Microbiology and Immunology, UBC). All *E. coli* strains were grown in H-broth (2.4g nutrient broth, 1.5g peptone, 1.5g sodium chloride, 0.3g glucose, 300ml water, pH 7.2). In addition, H-broth was used to perform dilutions for all *E. coli* strains and T4 phage. The T4 phage was stored in H-broth.

Phage Stock Preparation. *E. coli* C149 (wildtype) was inoculated into 25 ml of HB and incubated overnight at 37 ˚C in a shaking water bath. Subsequent overnight cultures were prepared the same way. The following day, molten PTA (6.5g tryptone, 4g sodium chloride, 1g sodium citrate, 1.5g glucose, 3.76g agar, 500 ml water, pH 7.2) was melted in a microwave at high power for approximately 1 minute and then placed in a 50˚C water bath. Next, supplied T4 phage stock (last titered in May 2000 to be 2 x 10^9 PFU/ml) was serially diluted in HB to 1x10^{-8} PFU/ml. Three milliliters of PTA was then dispensed into nineteen, 13 x 100 mm test tubes and placed in a 50 ˚C water bath. One hundred microlitres of *E. coli* C149 indicator cells was then added to each of the tubes containing PTA. Next, 100 ul was taken from each serial diluted T4 phage stock and also added to these tubes. Phage style mixing was employed to gently mix the phage and indicator cells in the PTA. After 15 seconds of mixing, the PTA suspension was poured on top of plates containing 20 mL of PBA (32.5g tryptone, 20g sodium chloride, 5g sodium citrate, 3.25g glucose, 37.5g agar, 2500 ml water, pH 7.2), previously warmed to 37˚C. It should be noted that serial dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were plated in duplicate, while serial dilutions of 10^{-6}, 10^{-7} and 10^{-8} were plated in triplicate. The plates were incubated overnight at 37˚C after which plate counts were performed to determine the phage titer. Only plates containing 30-300 plaque forming units (PFU)/plate were counted. The PTA from the plate containing the most plaques (without a complete clearing of the plate) was carefully scrapped off and transferred into 5 ml of HB. Four drops of chloroform was then added to lyse the *E. coli* host cells. This suspension was stored at 4˚C for 4 days to allow the phage to diffuse through the agar into the HB. On the fourth day, the entire suspension was centrifuged at 5000 x g for 5 minutes to remove the PTA. The supernatant was used as our T4 phage stock solution for the remainder of the experiment. The T4 phage stock solution was stored at 4˚C.

Phage Binding Assay. An overnight culture of each *E. coli* strain studied was grown in 25 mL of HB at 37˚C with shaking (180 rpm). The following day, each strain was diluted to obtain a turbidity of 0.4 OD_{660}, experimentally determined to be approximately equivalent to 4x10^8 cfu/ml (data not shown). The T4 phage stock was diluted to 2 x 10^6 pfu/ml using HB. Five minutes prior to infection, the *E. coli* C149 indicator cells, diluted T4 phage, and diluted *E. coli* strains in question were incubated in a 30˚C shaking water bath. At time 0 (T=0) 120 ul from the diluted T4 phage stock was used to infect 6 ml of the *E. coli* strain in question. Enough T4 phage was added to the overnight *E. coli* culture to produce an infection with a multiplicity of infection (MOI) of approximately 1:100,000. The infected culture was left shaking gently at room temperature for 8 minutes. Every 2 minutes, starting at T=0 (the point of infection), a 1 ml culture sample was taken and centrifuged at 5000 x g for 1 minute. The supernatant was removed and subsequently used in overlay plaque assays to determine the titer of unbound phage. The pellet was resuspended in 1 ml of HB and was divided into two 0.5 ml samples. One of these resuspended pellet fractions was used in overlay plaque assays to determine the titer of reversibly bound and internalized phage. The other 0.5 ml resuspended pellet fraction was treated with 50 ul of chloroform to artificially lyse the host cells and eradicate irreversibly bound phage. The chloroform treated sample was incubated at room temperature for 5 minutes and then used in the overlay plaque assay to determine the titer of reversibly bound phage. To ensure that the T4 phage did not pellet in the centrifugation step of the binding assay, pure samples of T4 phage were microfuged at 5000 x g for 5 minutes then plated in triplicate.

Overlay Plaque Assay. This method was employed to determine the titer of phage in each of the fractions obtained from the phage binding assay. Molten PTA was melted in a microwave at high power for approximately 1 minute and then placed in a 50˚C water bath. Three millilitres of the PTA was then dispensed into nineteen 16 x 120 mm test tubes and placed in a 50 ˚C water bath. Two hundred microlitres of *E. coli* C149 indicator cells were then added to each of the tubes containing PTA. Next, 100 ul and 20 ul samples were taken from each fraction of the phage-binding assay and added to separate tubes containing PTA and indicator cells. Phage style mixing was employed to gently mix the phage and indicator cells in the PTA. After 15 seconds of mixing, the PTA suspension was poured on top of plates containing 20 mL of PBA, previously warmed to 37˚C. The plates were incubated overnight at 37˚C after which plaque counts were performed to determine the phage titer for each of the mutant *E. coli* strains.

RESULTS

Bacterial and Phage Titer. The bacterial titer was determined to be 4 x 10^8 CFU/ml at 0.4 OD_{660}. The titer of the supplied T4 phage stock solution was determined to be 2 x 10^9 PFU/ml. The titer of our T4 phage after phage stock preparation was determined to be 8x10^9 PFU/ml. For our preliminary phage binding and overlay plaque assays, T4 phage was added to the overnight *E. coli* culture to produce an infection with an MOI of approximately 1:200,000. The initial phage titer (at T=0) was determined to be 1500 PFU/ml, which was 50% lower than we expected (Fig. 1). Suspecting that this may be due to an overestimation of the T4 phage stock solution, we doubled the volume of T4 phage added to the test cultures for our next assay, making the MOI approximately 1:100,000. For our subsequent assay, we obtained the desired phage titer of approximately 3200 PFU/ml (at T=0). A centrifugation assay was performed to determine the effects of centrifugation on T4 phage infectivity. The phage titers before and after centrifugation displayed a 10% difference. This suggests that the centrifugation process imparted no adverse effects on the phage's ability to infect the host cells.

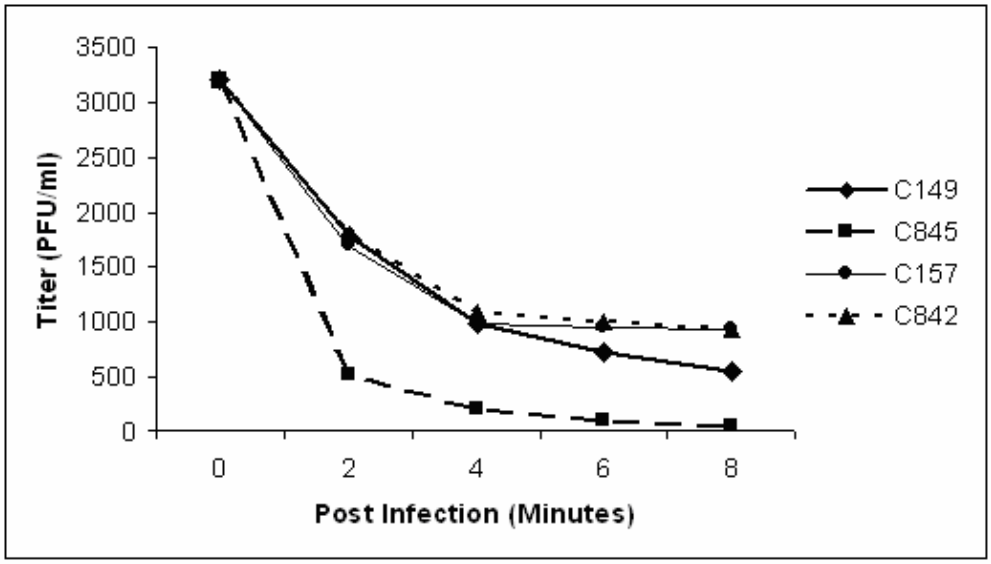

FIG. 1 The titer of unbound T4 phage in the supernatant fractions of the phage binding assay for *E. coli* C149 (wild type), C845 (LPS mutant), C842 (LPS mutant), and C157 (OmpC mutant).

The titer of unbound T4 phage. The titer of unbound phage was obtained from plate counts of the supernatant fractions. The phage binding assay suggests that despite mutations to LPS (strains C845 and C842) and OmpC (strain C157), T4 phage attachment was not hindered when compared to the C149 wildtype strain. T4 binding to the appropriate receptors of each *E. coli* strain is evident from the decline in phage titer across each time point (Fig. 1). When compared to the control and other mutant strains, the C845 LPS mutant strain consistently demonstrated the lowest phage titer at each time point (Fig. 1). This suggests that this strain had the highest binding affinity for the T4 phage. Interestingly, for the C842 LPS mutant and the C157 OmpC mutant, the unbound T4 phage titers in the supernatant were similar to the wildtype strain which suggests that these mutants had a similar binding affinity (Fig. 1).

The titer of detached reversibly bound T4 phage. The titer of reversibly bound phage released during the assay was obtained from plate counts of the fractions treated with chloroform. The stability of T4 phage attachment was highest for the C149 wildtype strain (Fig. 2). The release rate of reversibly bound phage for all strains approached an equilibrium 4 minutes post initial infection, represented by plateau in the trend (Fig. 2). Compared to the control or wildtype strain, all mutant strains had titers of reversibly bound phage that were at least one fold higher after the 2 minute time point. This observation indicates that the release rate of T4 phage from each mutant strain was significantly faster compared to that of the control, which is a characteristic of a decrease in the stability of T4 phage attachment. In addition, the increased T4 phage binding affinity observed for the C845 LPS mutant (Fig. 1) was compromised by a decreased phage binding stability (Fig. 2).

The titer of internalized T4 phage. In assessing the titer of internalized phage, plaque counts obtained from the pellet-chloroform fraction were subtracted from the pellet fraction at each time point. The C149 wildtype strain is observed to have an increase in internalized phage up until 6 minutes, after which the titer remains relatively constant (Fig. 3). Surprisingly, internalized phage was also observed in mutant strains C845, C842, and C157, but at very low titers at an average of 20 PFU/ml (Fig. 3).

Overlay plaque assay. An unusual observation was noted when determining the number of plaques formed 24 hours following the phage binding and overlay plaque assays. It was found that plaques were too faint to count or not visually apparent. After leaving the plates at room temperature for three more days, plaques were much more visible and counting was completed. It can be hypothesized that this lag in plaque formation was most likely due to a thin bacterial lawn 24 hours after the assay. The extra time allowed the bacterial lawn to thicken and made the plaques more visible for counting. The unusual thin bacterial lawn observed was due to the PTA not being properly cooled to 50^{o}C during the overlay plaque assay. Since previous studies report that *E. coli* is not tolerant to temperatures greater than 52˚C (10), improper cooling most likely killed the C149 indicator cells when they were added.

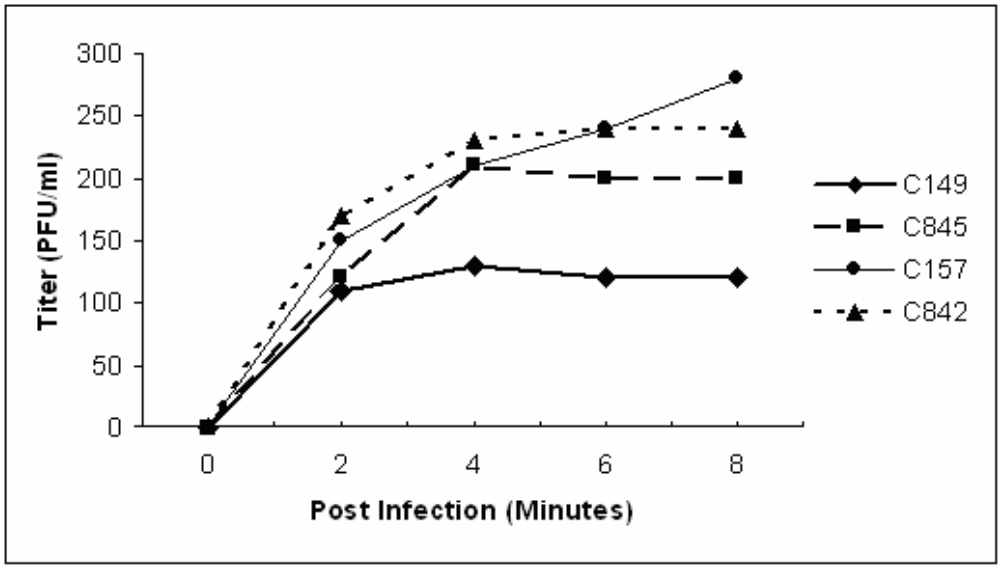

FIG. 2 The titer of detached reversibly bound T4 phage in the pellet fractions treated with chloroform of the phage binding assay for *E. coli* C149 (wild type), C845 (LPS mutant), C842 (LPS mutant), and C157 (OmpC mutant).

DISCUSSION

The titer of unbound T4 phage in the supernatant was determined for the wildtype and mutant strains every 2 minutes throughout the phage binding assay (Fig. 1). In general, we observed that the phage titer decreased throughout the assay for each individual strain (Fig. 1). This trend suggests that all strains were binding to the host cells and that the lack of infectivity observed by previous studies (9) with mutant strains C845, C842, and C157 can be attributed to problems in the viral infection process after initial phage binding. Previous studies have demonstrated that OmpC and LPS play an essential role in receptor function and infectivity (9,12) and it is likely that the sites where these mutations occurred may be non-essential to binding, but essential for a viral process that follows.

Surprisingly, the C845 LPS mutant showed improved T4 binding when compared to the C149 wildtype strain (Fig. 1). One possible explanation is that the LPS modifications for this strain may have contributed to increased binding interactions between the host cell and the phage. Reversible phage binding is weak and dependent on a variety of factors that vary from hydrogen bonding to hydrophobic and electrostatic interactions (4). We hypothesize that the modifications made to the LPS structure in C845 increased one or more of these interactions between the LPS and phage tail fibres.

Interestingly, the C842 LPS mutant and C157 OmpC mutant were found to share a similar binding affinity to T4 phage across each time point (Fig. 1). A possible explanation is that, in contrast to the C845 LPS mutant, modifications to the membrane components may not have influenced the binding interactions between the host cell and the phage.

The titer of detached reversibly bound phage was determined for each *E. coli* strain every 2 minutes throughout the phage binding assay (Fig. 2). Given that the error associated with phage enumeration is approximately 10% (6), the titer of reversibly bound phage for each mutant strain was not statistically different from one another. This suggests that each of the mutant strains had equal stabilities of reversible binding and hence similar release rates of T4 phage. All mutant strains in this study showed a decreased stability in T4 phage attachment when compared to the wildtype strain. More specifically, the titer of reversibly bound phage that have released in all mutant strains is at least one fold higher relative to that of the wildtype strain after the 2 minute time point (Fig. 2). An explanation for this observation is that the modifications made to the membrane components may have contributed to decrease the stability of reversible phage binding and hence increased the detachment rate of these reversibly bound phage. It is interesting to note that although strain C845 binds T4 phage at higher affinities, the binding is unstable and the phages detach easier compared to the other mutant strains. This suggests that modifications to LPS can influence both the affinities and stabilities of phage binding.

The titer of internalized T4 phage was determined for the wild type strain every 2 minutes throughout

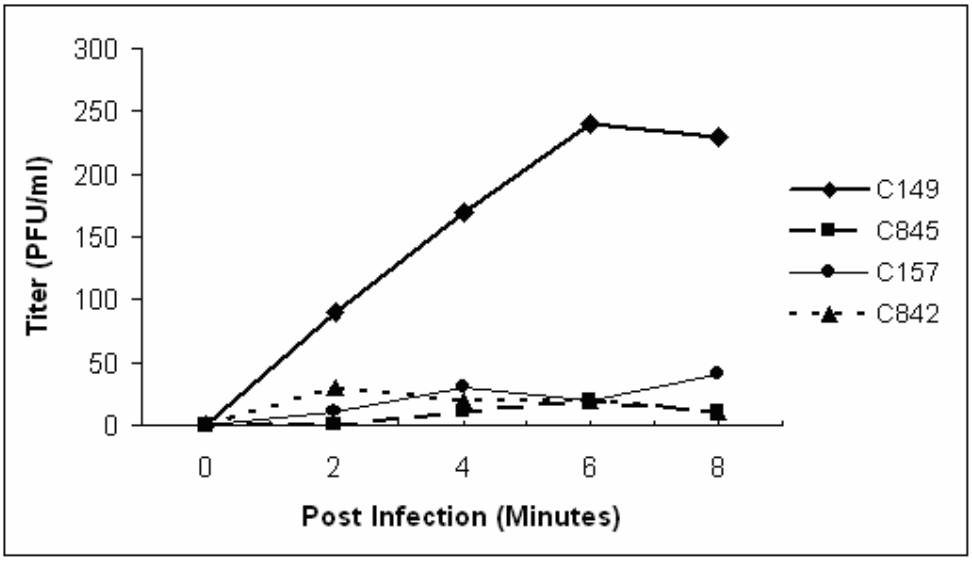

FIG. 3 The titer of internalized T4 phage during the phage binding assay for *E. coli* C149 (wildtype), C845 (LPS mutant), C842 (LPS mutant), and C157 (OmpC mutant).

the phage-binding assay (Fig. 3). An increase in the phage titer is observed up until 6 minutes which suggest that the phages are being internalized (Fig. 3). This is expected since previous studies have demonstrated that wild type strains of *E. coli* are permissive to T4 phage infection (5).

Surprisingly, internalized phage was also observed in mutant strains C845, C842, and C157 but at very low titers at an average of 20 PFU/ml (Fig. 3). This is unexpected since a previous study demonstrated that these mutant strains are non-permissive hosts for T4 phage infection (9). Suspecting that the previous study may have wrongfully determined the permissiveness of these mutant strains for T4 phage infection, we repeated the study with each of the mutant strains in question. Our results remained consistent with their study in that none of the mutant strains were permissive for T4 phage infection. This suggests that the phage are not internalized and that the phage titers observed are most likely reversibly bound phage that have slowly detached when the chloroform pellet fraction was prepared. This background will complicate the distinction between reversibly and irreversibly bound phage, but the magnitude of the loss in free phage and the poor release still suggest that the problem is subsequent to the initial reversible binding.

In this study, we report that LPS and OmpC play an essential role in one or more viral processes that follow binding. In addition, it was discovered that a mutation in LPS and OmpC for strains C842 and C157, respectively, did not alter their phage binding affinity but decreased their phage binding stability. Moreover, a mutation in LPS for strain C845 increased its binding affinity to T4 phage but decreased its binding stability. All these results suggest that modifications to LPS are capable of influencing both the affinities and stabilities of phage binding.

FUTURE EXPERIMENTS

In this study, we report that LPS and OmpC play an essential role in one or more viral processes that follow binding. Such processes may vary from phage DNA injection to viral gene expression, assembly, or release. An interesting future experiment could investigate whether OmpC and LPS play a role in phage DNA delivery in *E. coli*. This study would involve infecting OmpC and LPS mutants using T4 phage containing ^{14}C-radiolabeled DNA. If radiolabeled DNA are detected within these cells, it would suggest that OmpC and LPS are involved in one or more viral processes after phage DNA injection. On the other hand, if no radiolabeled DNA is detected, it would suggest that OmpC and LPS are involved in phage DNA injection.

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