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Assessing binding ability and stability of T4 bacteriophage to *Escherichia coli* with mutations in the T4 bacteriophage receptors

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Previous studies have demonstrated the importance of LPS and OmpC for bacteriophage T4 infectivity in *Escherichia coli* by showing that mutations in either LPS or OmpC result in altered infectivity relative to wild type strains. However, it still remains unclear whether the mutations in LPS and OmpC result in altered infectivity because of impaired bacteriophage binding, or some downstream viral process that follows. To investigate this further, phage binding and overlay plaque assays were performed on *E. coli* K12 strains C149 (wild type), C157 (OmpC$^-$), and C843, C846, and C848 (LPS mutants). In this study we provide support for previously described reports of altered infectivity immediately following infection of these mutants, and illustrate that these infectivity trends do not necessarily remain stable over time. Previously reported to result in a complete lack of infectivity, we provide evidence that C157 (OmpC$^-$) becomes susceptible to T4 infection soon after the initiation of infection. The increased infectivity of C848 and C843 and decreased infectivity of C846 appear to be a result of altered binding abilities.

T4 bacteriophage, a member of the T-even phage group, is an efficient infectious agent of *E. coli* (2). Two cell surface components of *E. coli* serve as receptors for bacteriophage with the T-even type structure (11). The first component is lipopolysaccharide (LPS), which is located exclusively in the outer membrane of gram negative bacteria (5). The second bacterial component serving as a bacteriophage receptor is the outer membrane protein OmpC (11), which is involved in sensing and responding to changes in environmental osmolarity in *E. coli* (8).

It has been recognized for over 50 years that bacteriophage attachment to its host cell occurs in two distinct phases: a reversible stage preceding an irreversible stage (4). The reversible adsorption, characterized by its transient nature, occurs when the long phage tail fibers attach to OmpC of the K12 *E. coli* surface (7). While reversible attachment still allows the phage to move over the outer membrane surface, OmpC recognition by the tail fibers transmit a signal to the baseplate of the phage in the form of a conformational change (7). This fiber-binding step thus activates the baseplate which permits baseplate recognition of LPS on the surface of K12 *E. coli* that characterizes the irreversible binding stage (7).

To further characterize which particular aspects of the LPS and OmpC structures were required for infection by bacteriophage T4, Wang and Lin (9) tested various *E. coli* strains with mutations in the structure of LPS, or deletion of OmpC. They found that there was no infectivity in the OmpC$^-$ mutant, indicating a requirement for OmpC. They also found increased infectivity in two LPS mutants relative to the wild type strain, and decreased infectivity for numerous other LPS mutants. We selected the OmpC$^-$ strain (C157), the two LPS mutants that illustrated increased infectivity (C843 and C848) and an LPS mutant with decreased infectivity (C846). We measured the titer of unbound phage, the titer of reversibly bound phage, and the titer of total bound phage for each *E. coli* mutant.

The purpose of our experiment was to confirm the infectivity results of Wang and Lin (9) and to ascertain whether the increased or decreased infectivity observed for each mutant was due to altered binding ability, as measured by the titer of unbound phage. Should altered infectivity be correlated with altered binding ability or stability, the mutation in either LPS or OmpC would appear to be important in the attachment of phage to the bacterium, rather than another downstream process involved in viral replication or release.

MATERIALS & METHODS

Bacterial & Phage Strains. T4 phage, and *E. coli* K12 strains as described (9, 10), were from the MICB 421 Teaching Lab frozen stock (Department of Microbiology & Immunology, UBC, Canada). The following K12 strains were used in this study: C149 (wild type), C157 (OmpC$^-$), and C843, C846, and C848 (LPS mutants). A more complete listing of phenotypes and LPS structures of these strains appears in the appendix (9, 10). All *E. coli* strains were grown in H medium, which was prepared as previously described (6). H medium was also used for dilutions and for storage of T4 phage.

Phage Stock Preparation & Enumeration. H medium was inoculated with *E. coli* C149 and incubated overnight at 37°C in a shaking water bath (approximately 180 rpm). PTA medium (13.0 g Tryptone, 8.0 g NaCl, 2.0 g sodium citrate, 3.0 g glucose, 7.2 g agar, 1.0 L distilled water, pH 7.2) was melted in a microwave and placed in a 50°C water bath. The supplied T4 phage stock was serially diluted in H medium up to 10^{-8}. 100 µl from each serial diluted T4 phage stock and 100 µl *E. coli* C149 indicator cells were added to 3 ml PTA medium. Phage were mixed before pouring the PTA mixture

onto plates containing 20 ml of solid PBA medium (13.0 g Tryptone, 8.0 g NaCl, 2.0 g sodium citrate, 1.3 g glucose, 15.0 g agar, 1.0 L distilled water, pH 7.2). Serial dilutions were plated in duplicate. The plates were incubated overnight at 37°C. PTA from four plates containing 30-300 plaques was scraped off and transferred into 10 ml of H medium. Eight drops of chloroform were added to this suspension (to sterilize the suspension). This suspension was incubated at 4°C for four days. The suspension was then centrifuged at 5000 × g for 10 minutes at room temperature, and the supernatant containing phage was collected and stored at 4°C, and used as a stock for all experiments. This phage stock was serially diluted (tenfold) in H medium up to 10^{-8}. These dilutions were plated as described above to determine the phage titer.

Phage Binding Assay. Overnight cultures were prepared as described above for each *E. coli* strain. Cultures were subsequently diluted in H medium to a turbidity of 0.4 OD_{660}, which was shown in a previous study to correspond to 4×10^8 CFU/ml (1). Prior to infection, the diluted *E. coli* cultures were placed in a 30°C water bath. The phage stock was diluted to a concentration of 2×10^6 PFU/ml in H medium. At the beginning of the experiment, 130 µl of diluted phage stock was added to 6.5 ml of each culture to establish a multiplicity of infection (MOI) of 0.0001. The infected cultures were mixed briefly using phage style mixing. Every 3 minutes, up to 12 minutes, starting at the onset of infection (t=0), 1 ml samples were removed and centrifuged at 5,000 × g for 1 minute. It has been previously shown (1) that centrifugation does not greatly affect T4 phage infectivity. The supernatant was collected to determine unbound phage titer using the overlay plaque assay. The pellet was resuspended in 1 ml of H medium, split into two 0.5 ml fractions and kept on ice until plating. One of these fractions was used in the overlay assay to determine the titer of total bound and internalized phage. 50 µl of chloroform was meant to be added to the second pellet fraction to determine the titer of reversibly bound phage. However, the chloroform-treated pellet fraction was not included in the experiment for logistical reasons.

Overlay Plaque Assay. Twenty and one hundred microliters of each of the samples obtained from the supernatant or either pellet treatments were each used for the overlay plaque assay. Melted PTA medium was aliquotted to tubes (3 ml per tube) and placed in a 50°C water bath. These samples and 200 µl *E. coli* C149 were added the tubes of PTA medium. After brief phage style mixing, this mixture was poured onto plates containing PBA. After PTA solidification, plates were incubated overnight at 37°C. Plates containing between 30 and 300 PFU were enumerated to determine phage titer.

RESULTS

Bacterial and Phage Titer. Based on a previous study (1), the bacterial titer was determined to be 4×10^8 CFU/ml at 0.4 OD_{660}. The titer of the T4 phage stock that we prepared was determined, by PFU counts, to be 2.7×10^7 PFU/ml.

Overlay plaque assay. As previously reported by others using this assay (1), the bacterial lawn observed after overnight incubation was very thin, making plaques difficult to count. This phenomenon was observed despite taking care to use PTA medium that was no warmer than 50°C, as warmer medium may adversely affect the survival of the indicator cells. Furthermore, many of the plates had PTA that had solidified prematurely, introducing cracks and lumps into the top layer of agar. This made it difficult to differentiate between plaques and imperfections in the agar surface, resulting in uncertain phage enumeration.

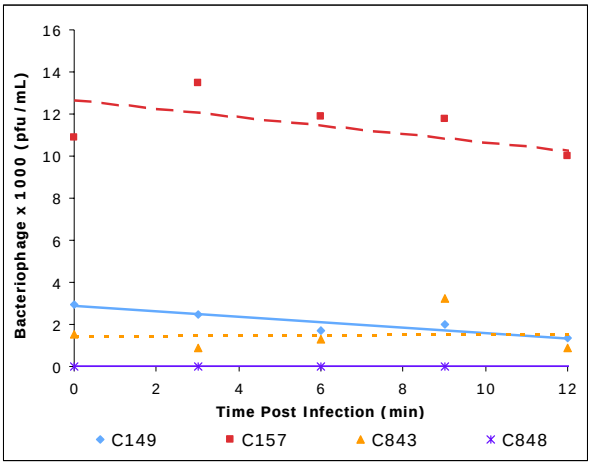

Fig. 1. Titer of unbound T4 phage in the supernatant fractions of the phage binding assay for *E. coli* C149 (WT), C157 (OmpC$^-$), C843 (LPS mutant), and C848 (LPS mutant). C846 (LPS mutant) was not included in this figure due to inability to distinguish plaques; it is likely that these plates showed complete clearing.

Titer of unbound T4 phage. The number of unbound phage is indicated by the phage titer in the supernatant fractions of the phage binding assay. The results indicate that all mutants, with the exception of C157 and perhaps C846, had increased susceptibility to phage binding compared to the wild type strain, C149 (Fig. 1). C149 levels of unbound phage decreased over time from 2955 PFU/ml to 1340 PFU/ml. A decrease to approximately half the initial phage titer implies a corresponding increase in phage binding. C157 had more unbound phage than the wild type (C149). The level of unbound T4 phage for C843 was consistently less than the wild type (C149), implying the possibility of increased infectivity compared to wild type. C848 did not present any plaques in the supernatant fraction, which could indicate complete binding. It is also possible that the lack of PFU in this fraction is due to some form of experimental error. C846 was not included in Fig. 1 due to indications of complete clearing, which makes the phage titer impossible to accurately determine. However, the phage titer for C846 would be greater than 14,000 which falls within the countable range as observed for C157 (Fig. 1).

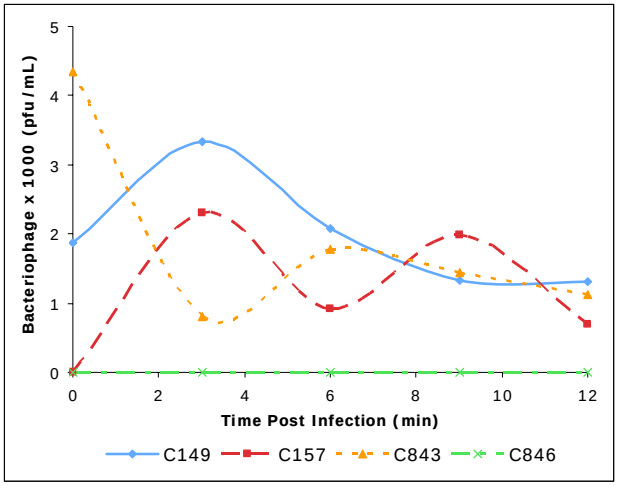

Fig. 2. Titer of total bound T4 phage in the pellet during the phage binding assay for *E. coli* C149 (WT), C157 (OmpC$^-$), C843 (LPS mutant), and C846 (LPS mutant). C848 was not included in this figure due to inability to decipher plaques; it is likely that these plates showed complete clearing.

Titer of total bound T4 phage. Plate counts of the untreated pellet fraction provide a measure of reversibly plus irreversibly bound phage. Wild type bacteria (C149) showed an initial increase in PFU, followed by a gradual decrease. The OmpC$^-$ strain began with no binding in the pellet fraction, followed by alternating trends of increased and decreased binding to cells. The LPS mutant C843 showed initial high binding, with a subsequent drop and eventual equilibrium. C846 showed no infectivity, as determined by lack of PFU in the pellet fraction. The lack of infectivity for the C846 pellet fraction is consistent with the high supernatant titer and poor binding to the cells (Fig. 1). C848 was not included in Fig. 2 because it produced no distinct plaques. However, due to the lack of confluent bacterial lawn, and the appearance of a few distinct colonies, it seems likely that the lack of plaques was due to complete clearing. Complete clearing would be consistent with the high initial binding predicted by the lack of phage in the supernatant at time zero (Fig. 1).

DISCUSSION

Comparison of the phage output from the phage binding assay to the input phage value (40,000 PFU/ml) indicates a significant discrepancy. For example, at time zero the wild type cells (C149) produced approximately 3000 PFU/ml in the supernatant fraction, and 2000 PFU/ml in the pellet fraction. Therefore, with an input phage value of 40,000 only 5,000 phage produced plaques. This discrepancy indicates a source of experimental error that may include overestimation of phage stock or incomplete plaque detection on plates from the phage binding assay caused by a lack of confluent bacterial lawn.

The range and trend of unbound phage titer for wild type (C149) agrees with previous studies (1). However, total bound phage, as measured by overlay assay of the untreated pellet fraction, increased up to t=3 only to gradually decrease to its original level from t=0. This trend was unexpected based on previous observations (1) where the numbers of plaques in the pellet fraction increased from t=0 and eventually leveled off. However, this unexpected trend may be a result of measurement uncertainties, rather than a reflection of a real biological phenomenon.

The OmpC$^-$ mutant (C157) produced unbound phage titers that were on average fourfold greater than wild type (C149) (Fig. 1). While this trend seems consistent and supports previous literature findings that OmpC is a phage-binding site on bacteria (3), the exact magnitude of phage titer may be inaccurate due to the poor agar quality of the C157 supernatant plates. The total bound phage was initially very low, correlating with the significantly higher phage titer from the supernatant fraction compared with wild type. The initial low phage titer from the pellet fraction also agrees with the lack of infectivity immediately following infection as reported previously (9). However, this lack of infectivity is not seen after t=0 (Fig. 2), a result that suggests that OmpC is not an absolute requirement for binding. However, no conclusion can be drawn about whether OmpC is essential for infection, since bound phage could subsequently unbind and infect indicator cells. Should the total bound phage titer in our experiment represent reversibly bound phage only, OmpC would also appear to be important in some viral replication process downstream of binding.

Based on previous literature findings (7) we would not expect to see any infectivity for the OmpC$^-$ (C157) mutant because of the importance of OmpC in reversible binding. However, some infectivity, albeit limited, was reported recently (1). With the possibility of experimental error eliminated, this would suggest that there is either an alternate unidentified reversible attachment site for T4 bacteriophage or that LPS can partially compensate for absence of OmpC. In the case of *E.coli* B, wild type LPS alone is sufficient for phage attachment and infectivity (11).

Since the unbound phage titer for C157 continues to decrease over time, a corresponding increase in phage titer within the pellet fraction is expected. However, an increase in total bound C157 was not consistently seen over the time course of 12 min (Fig. 2). This discrepancy could be explained by an increase in irreversible binding that did not result in productive infection. For example, OmpC has been implicated in

structural stability of the membrane facilitating phage DNA injection (3). Therefore, it is possible that the absence of OmpC impedes DNA injection into the cytoplasm resulting in fewer productive infections.

The phage titer for the pellet fraction of the LPS mutant C843 was twice that of wild type (C149) immediately following infection at t=0 (Fig. 2). This increase in bound phage immediately following infection confirms previous findings (9). However, the phenomenon of increased infectivity for C843 appears to be short-lived since the bound phage titer was more variable following t=0, decreasing before eventually reaching equilibrium. This may reflect an increased frequency of binding but a less stable interaction of the C843 receptors for T4. Should this be the case, an increase in reversible binding would be expected. The amount of unbound phage for LPS mutant C843 remained fairly constant throughout the course of infection, and began as half that of the wild type (Fig. 1). This lower titer of unbound phage explains the increased total bound phage to C843 at t=0. The lack of high total bound phage over time is likely a result of increased reversible binding.

C846, another LPS mutant, demonstrated severely impaired phage binding, as illustrated by the absence of plaques for the pellet fraction as well as the apparent clearing of indicator cells by the supernatant fraction. We believe that the plates containing the supernatant fraction demonstrated complete clearing because of the presence of several small colonies on these plates, which likely represent phage-resistant colonies, a characteristic of complete clearing on a plate of indicator cells. This finding of impaired phage binding confirms an earlier publication (9) that C846 is T4 phage resistant, and our findings suggest that this is due to impaired binding of T4 to the LPS receptor.

C848, the final LPS mutant, showed complete clearing in the pellet fraction and a complete lack of plaques in the supernatant fraction, suggesting extremely high binding ability of phage to this mutant type. However, due to chance alone, it seems unlikely that all phage could have bound to the cells by t=0. Due to the contribution of poor plate quality to plaque counting difficulties, it is difficult to have complete confidence in this data. However, the complete clearing in the pellet fraction does provide support for earlier reports of increased infectivity (9), and the low or no phage titer in the supernatant fraction implicates binding as a component of increased infectivity.

Total bound phage titer for the wild type *E. coli* (C149) did not increase over time as expected (1) for unknown reasons. Measurements of unbound phage for the $OmpC^-$ mutant quantitatively support the assertion that OmpC plays an important role in phage binding, as previously described (11). While the OmpC knockout (C157) illustrated significantly impaired phage binding, as reflected by the high titers of unbound phage, infectivity at later time points was seen in the pellet fraction, representing total binding. Since the total bound phage titer represents both irreversibly and reversibly bound phage, it is possible that all productive infections for the C157 pellet fraction represent reversibly bound and detached phage that produce plaques by infecting wild type indicator cells rather than the C157 cells. Therefore, our data does not refute the requirement of OmpC for productive infection as reported earlier (7), but does suggest that perhaps a molecule other than OmpC may mediate some degree of unstable binding between phage and bacteria, accounting for the infectivity seen in our total bound pellet fraction.

In the LPS mutant C843, the lack of PEEN appears to have increased binding frequency but also decreased stability compared to wild type *E. coli*. Although both LPS mutants C846 and C848 lacked a terminal sugar group, they produced two distinct binding profiles. Lack of terminal β-glucose and glucose-galactose groups impaired T4 bacteriophage binding as seen in C846 LPS mutant. However, lack of terminal β-glucose alone increased infectivity and binding affinity as seen in C848. Technical difficulties with some experiments made definite conclusions difficult to make, and key protocol changes are necessary for collection of more illuminating results.

FUTURE EXPERIMENTS

Since we observed significant variation between time points, it would be useful to have 0.5 min intervals between time points (instead of 3 min), in order to help elucidate changing trends, particularly at the beginning of the time course, so that the initial trend can be seen. Another potential future experiment would be to repeat the binding assays using additional LPS mutants in order to identify other components of LPS that may be important for bacteriophage binding. Binding stability of these mutants could also be investigated by treating a portion of the pellet fraction with chloroform, although preliminary attempts to utilize this method proved unsuccessful. Finally, it would be useful to obtain $OmpC^-$ strains having LPS mutations in addition, to determine whether parts of the LPS structure are involved in compensation of binding ability in the absence of OmpC.

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REFERENCES

1. **Chung, W., J. Siu and K. Tanner.** 2005. Roles of Lipopolysaccharide and the Outer Membrane Protein OmpC on Bacteriophage T4 Infectivity in *Escherichia coli*. J. Exp. Microbiol. Immunol. **7**:62-67.
2. **Douglas, J.** 1975. Bacteriophages. Chapman and Hall, London.
3. **Furukawa, H., T. Kuroiwa, and S. Mizushima.** 1983. DNA injection during bacteriophage T4 infection of *Escherichia coli*. J. Bacteriol. **154**: 938-945.
4. **Goldberg, E.** 1983. Recognition, attachment, and injection, p. 32-39. *In* C.K. Mathews, E.M. Kutter, G. Mosig, and P.B. Berget (ed.), Bacteriophage T4. American Society for Microbiology, Washington, DC.
5. **Madigan, M.T., J.M. Martinko, and J. Parker**. 2000. Brock biology of microorganisms, 9th ed. Prentice Hall, Upper Saddle River, NJ.
6. **Miller, J.H.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbour Laboratory Press, Plainview, N.Y.
7. **Mosig, G. and F. Eiserling.** 1988. Structure and initiation of infection, p. 538-542. *In* R. Calendar (ed.), The Bacteriophages: Volume 2. Plenum Press, New York NY.
8. **Pratt, L.A., W. Hsing, K.E. Gibson, T.J. Silhavy.** 1996. From acids to *osmZ*: multiple factors influence synthesis of the OmpF and OmpC porins in *Escherichia coli***.** Mol. Micro. **20**: 911-917.
9. **Wang, A. and K. Lin.** 2001. Effect of mutations in the outer membrane components on bacteriophage T4 adsorption to *Escherichia coli*. J. Exp. Microbiol. Immunol. **1**:47-53.
10. **Yethon, J., D. Heinrichs, M. Monteiro, M. Perry and C. Whitfield.** 1998. Involvement of *waaY*, *waaQ*, and *waaP* in the modification of *Escherichia coli* lipopolysaccharide and their role in the formation of a stable outer membrane. J. Biol. Chem. **273:** 26310-26316.
11. **Yu, F. and S. Mizushima.** 1982. Roles of lipopolysaccharide and Outer Membrane Protein OmpC of *Escherichia coli* K-12 in the receptor function for bacteriophage T4. J. Bacteriol. **151**: 718-722.