The Dynamics of Bacteriophage T4 Binding to *Escherichia coli*

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The binding dynamics of bacteriophage T4 to wild type, OmpC and an LPS mutated strain of *Escherichia coli* were assessed. Previous authors determined that bacteriophage T4 was inhibited from infecting the OmpC strain and showed decreased infectivity in the specific LPS mutants. To determine why infectivity rates for the mutants were decreased, we used a phage-binding assay to establish whether was limited due to a lack of bacteriophage binding or due to inhibition of injection of bacteriophage DNA into the cell. Unfortunately, due to unknown reasons, the binding assays were not successful. Secondary experiments were conducted to deduce the possible reasons for failure of the assay. Our results indicated that the failure might have been due to an insufficient multiplicity of infection or to too dense a lawn of indicator cells, which could inhibit plaque formation.

The T4 bacteriophage, a member of the T-even phage group, is an efficient infectious agent of *Escherichia coli*. Its structure consists of a DNA-containing head, a tail, and a baseplate, which is attached to both long and short tail fibers (9). The viral replication cycle is initiated with the reversible binding of the phage to the host cell through its long tail fibers (8, 4). Following this weak binding, the short tail fibers are then able to irreversibly bind to the bacteria, efficiently anchoring the virus to the host cell’s outer membrane (8). Once this event has occurred, the phage base plate can penetrate through the cell’s cytoplasmic membrane and subsequently insert its genome into the host cytoplasm.

Previous studies have noted the importance of both lipopolysacharride (LPS) and the outer membrane protein, OmpC, in the binding of phage to *E. coli* (8, 9). One particular study found that the addition of glucose derivatives N-acetylgulosamine and α-methylglucoside inhibited T4 phage adsorption (8). Furthermore, *E. coli* strains lacking OmpC as well as strains with variations in their LPS structures were shown to be more resistant to infection by T4 phage (8). Although these studies have helped to identify that both LPS and OmpC are required for initial phage binding, it is unclear if these two outer membrane elements have a role in attachment of the phage baseplate and thus ejection of viral DNA into the host. Therefore, the purpose of this experiment was to identify if LPS and OmpC are involved in both initial phage binding as well as insertion of viral DNA into the host cytoplasm. We hypothesize that both outer membrane constituents are necessary for phage attachment.

**METHODS AND MATERIALS**

**Bacterial and Phage Strains.** *Escherichia coli* strains B23, C157 (OmpC) and C847 (LPS mutant), used in this experiment, were provided by Dr. W. Ramey of the Department of Microbiology and Immunology of the University of British Columbia. Both strains were described previously (8). T4 phage was donated by Karen Smith also of the Department of Microbiology and Immunology of the University of British Columbia. All *E. coli* strains were grown in Luria Broth. The T4 phage was stored in Luria Broth.

**Growth Media.** The Luria-Bertani (LB) broth media and phage top agar were made according to instructions given in the Microbiology 421 lab notebook. The Tryptic Soy Broth (TSB) was made as follows Bacto-Tryptone (17.0 g), Bacto-Soytone (3.0 g), Bacto-Dextrose (2.5 g), Sodium Chloride (5.0 g), Dipotassium Phosphate (2.5 g), water to 1000 mL, pH 7.2. Modified LB broth contained the following: Tryptone(13g), sodium chloride (8g), sodium citrate (2g), glucose (1.3g), agar (15g), water to 1000ml, adjust pH to 7.2. To make this broth, add the first three ingredients into a flask and fill to 1000 ml. From this, remove 100 ml and add the 1.3g of glucose to the 100ml. Add the agar to the 900ml mixture and autoclave both mixtures. Mix the two flasks together after autoclaving. Top agar can be made the same way except using an agar concentration 0.6%.

**Phage Stock Preparation.** *Escherichia coli* B23 was inoculated into 25 ml of tryptic soy broth (3) and grown overnight with shaking at 37°C. The stock bacteriophage T4 (3 x 10⁶ pfu/ml) was serially diluted using Luria Broth to a dilution of 10⁻⁵. One hundred microliters of each dilution was added to 0.5 ml of *E. coli* B23 indicator cells and this mixture was left at room temperature for 30 minutes. After incubation, 2.5 ml of phage top agar was added to each dilution/indicator cell mixture and plated. The plates were incubated overnight at 37°C. The next day, the plate the plate with the 10⁻⁵ dilution displayed enough plaques so that there wasn’t clearing but the plaques were running into each other. Fifteen milliliters of Luria Broth was added to this plate and the top of it was slightly scraped. This plate was put back into 37°C incubator overnight. Ideally, this plate should have been put into the 4°C refrigerator, but this does not seem to have had any effect on the titer. The following day, the phage, Luria Broth mixture was removed from the top of the plate and centrifuged at 5000 x g for 5 minutes. The cleared supernatant was collected and used as “amplified phage” stock. This stock was titred by making serially dilutions and plating using the protocol mentioned above.

**Phage Binding Assay.** An overnight culture of the *E. coli* strain in question was grown in 25 ml of Luria Broth at 37°C with shaking (180 rpm). Five minutes prior to infection, both the overnight *E. coli* culture and the T4 phage were incubated in a 30°C shaking water bath. T4 phage was added to the overnight *E. coli* culture to produce an infection with an MOI of approximately 1:33,000. The infected culture was incubated in a
30°C shaking water bath for 12 minutes. Every 3 minutes, starting at T = 0 (the point of infection), a 1 ml culture sample was taken and centrifuged at 5500 x g for 1 minute. The supernatant was removed and subsequently used in overlay plaque assays to determine the concentration of unbound phage. The pellet was resuspended in 1 ml Luria Broth and was divided into two 0.5 ml samples. One of these resuspended pellet fractions was used in overlay plaque assays to determine the concentration of bound phage to susceptible host cells. 200 µl of chloroform was added to the other 0.5 ml resuspended pellet fraction. The chloroform sample was incubated at room temperature for 5-7 minutes and then used in the plaque overlay assay to determine the amount of phage bound to host cells, but unable to infect.

**Overlay Plaque Assay.** This method was employed to determine the concentration of phage in the samples. Molten phage top agar was melted in a microwave at high power for approximately 1 minute, cooled to about 50°C, and then placed into a 50°C water bath. Serial dilutions of the phage samples were prepared using Luria Broth to ensure that between 30-300 plaques would be present when plated. For the supernatant, dilutions were made up to 10³; dilutions for both pellet samples were made up to 10². One hundred microliters of each of the dilutions (supernatant, pellet, and pellet with chloroform) was added to 0.5 or 0.3 ml of E. coli B23 indicator cells. The pellet/chloroform and supernatant-indicator cell mixtures were incubated at room temperature for 30 minutes before addition of top agar and plating. The pellet samples (without chloroform) were plated immediately after the addition of indicator cells. Three milliliters of phage top agar was added to the sample/indicator cell mixture and mixed in 13 x 100 mm tubes using phage style mixing. The mixture was quickly poured onto plates containing either LB bottom agar or modified LB bottom agar. These plates were incubated at 37°C for 24 hours after which plaque counts were performed.

**RESULTS**

We performed several phage-binding assays with the different strains of *E. coli*: B23, C157 and C847, but were unable to obtain meaningful results for most of them. In the first assay on *E. coli* B23, we obtained plaque counts for the time point supernatants that were exactly the opposite of what we expected to see (Table 1). For the pellets of the time point, all plates had plaques that were too numerous to count (TNTC). *Escherichia coli* C157 was the next strain to be tested in the assay. Only one set of plates for this strain fell within the statistically significant range of 30 to 300 plaques, some plates were completely cleared of phage, and some had no plaques present at all, overall indicating a problem with some part of the technique. Similar results were obtained for a second trial of *E. coli* B23. Plates with complete confluent lawns lacking any plaques, plates completely cleared by phage, and plates that appeared to lack both indicator cells and phage were obtained for this strain. We had no plates that were in the 30 to 300 plaques per plate range. We performed a second trial with *E. coli* C157, and again obtained only plates with confluent lawns lacking any plaques.

At this point, due to the problems encountered early on, we decided to again titrate the T4 bacteriophage amplified stock that we had been using to infect our cells. We did this using the *E. coli* C157 strain, and did not obtain any plaques on the plates. This lead us to believe that there may have been a problem with the viral stock, and we performed a secondary experiment to verify if this was the case. We tested three different stocks of phage: the amplified phage that we had been using all along (the “amplified phage”), an alternative stock of phage provided to us (the “new phage”), and the same stock of phage that we had used to amplify our own stock, that had come straight out of the freezer (the “stock phage”). All three strains of virus were able to infect the host and give proper plaque results, which indicated that the phage stock was fine. We also verified that the indicator cells we were using, *E. coli* B23, as well as the two other strains, were susceptible to phage infection. Further, we tried varying the procedure by adding tryptophan, which phage require in order to infect, to the phage top agar on some of the plates, and found that this did not play a role in whether or not phage were able to produce plaques.

The phage-binding assay was repeated for the *E. coli* C157 and C847 strains. We obtained no plaques on any of the plates, including the supernatant samples or the pellet samples, for either of these two strains. Confluent lawns were produced, but no plaques.

**DISCUSSION**

Our results indicate problems with the phage binding assay protocol, yet we know that the *E. coli* B23 indicator cells we were using are able to be infected by the T4 phage, and that the T4 stock that we had amplified was able to infect cells. This was indicated by the secondary experiment that we performed, as all cultures showed ampie signs of clearing. There was no discernible reason why the cells grown up in culture for the experiments would be any different some the cells used in these tests. Another possible problem was the composition of the media that we were using. The T4 phage has a requirement for tryptophan, which acts as a co-factor for adsorption (1). The media we were using for several of the trials did not contain an adequate amount of this compound. We had originally thought that the shortage of tryptophan might have been the reason for the absence of phage adsorption. However, the secondary experiment that we performed confirmed that whether or not tryptophan was added to the top agar, phage could still be produced. This is because there are a number of other compounds that can be used in the same role, and at least one of these is usually present in rich media. Examples of this are L-tyrosine or L-phenylalaline. With all these verifications, it appears that all parameters were in order and all the necessary factors for obtaining
reliable results were accounted for. This means that there was some difference during the experiment itself that was responsible for the lack of results.

It should first be considered that phage growth and adsorption is affected by a number of other environmental factors such as cell size, age, shape, growth rate and density (2). For instance, the higher the growth rate of the cell, the higher the level of phage adsorption. Greater cell surface areas yield a higher adsorption rate for obvious reasons. These parameters vary from culture to culture, but given the similar times of incubation and growth conditions, it is unlikely that any of them would vary widely enough to prevent adsorption all together. In addition, it should be remembered that there was no evidence of phage in the supernatant cultures, suggesting that the problem was not merely that the phage was not being adsorbed, but that either they were not surviving or not enough phage was being added. However, for each of the cultures tested, the number of phage added was calculated to equal the number added in the first trial making the number of cells/phage constant throughout the experiment.

It is believed that one of the main factors affecting plaque formation was the density of the indicator cells on the plates. All of our plates had a very dense confluent lawn. If the confluent lawn is too thick, infection by phage may be inhibited or result in very small plaques. Since we did observe very small plaques in our last set of experiments, this may have been a factor. We attempted to decipher whether this really was a factor affecting phage growth and tested infectivity using 0.5 ml (what we were using all along) of indicator cells. During this trial, plaques were observed however, when we repeated our protocol using 0.3 ml of indicator cells, no plaques were observed suggesting that this was not a factor. However, the day those trials were done, the culture of indicator cells was not as dense as the other days we performed the experiment due to insufficient aeration. This means that fewer cells were used in the successful trials then in the other experiments so the indicator cell density might still be a factor here. Since the dynamics of phage infection in plaque assays is basically a race between lawn growth and phage infection, the presence of too many indicator cells may have limited growth and infection before the plaques became visible. Further testing is needed to make more concrete conclusion.

Other experimental variables that could potentially play a role include the length of time the dilutions sat, the type of media that the amplified phage was stored in, how the top agar was melted, and the multiplicity of infection used to infect the cells. Experiments were performed to determine whether the lengths of time dilutions were kept before plating affected the results. Our results indicate that this was not an issue. The amplified phage was stored in Luria Broth, which may contain factors detrimental to phage survival. Furthermore, since the amplified phage was a crude cell lysate, there may be enzymes or other factors in the mixture that kill bacteriophage but the “amplified phage” stock was stored for at least two weeks without a significant drop in titre so this is not likely to be the cause.

The top agar was kept at the recommended 50°C following melting, however it may have been used too soon after removing from the microwave, having an adverse effect on phage survival. However, the tests took several hours to complete so even if the melted agar was too warm, the top agar in the later experiments had time to maybe cool to 50°C. However, the agar was usually heated to boiling in the microwave and then cooled. Since the day we performed some of the secondary tests on the “Amplified phage,” “New Phage,” and “Stock Phage” the top agar was boiled to melting and not microwaved, it was considered that this might have had an effect. Further testing concluded that means of melting the top agar was melted had no effect on the phage infectivity.

Ideally, multiplicity of infection should be lower so that single cell infections are guaranteed. It is possible that our levels of phage were too low, and the phage could not replicate sufficiently to infect the cell population. According to Abedon (1), if a lower multiplicity of infection is used, the lysis of infected cells can be inhibited. If there is no lysis of the infected cells, then there is no phage available in the extract to infect the indicator cells and induce plaque formation. Therefore, one will see an increase in cell number in infected culture as time passes, which may account for the presence of confluent lawns. However, whenever a plaque develops in a lawn, it develops at a very low multiplicity of infection since a plate with one plaque started with one phage and approximately ten million host cells in the overlay. In addition, some of the tests should have residual phage that would only infect hosts in the overlay. This means that the problem was probably not due to a low multiplicity of infection.

**FUTURE EXPERIMENTS**

Future experiments should explore how the MOI and/or the density of indicator cells play a role in infectivity of *E.coli*. To do this, we propose that the protocol be repeated using a lower density of indicator cells, achieved by diluting the culture before use to an optical density of 0.4 when measured at a wavelength of 660nm. Further, the MOI should be increased to at least 1:1000. Following optimization of these parameters, the assay should be repeated with these.
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REFERENCES