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T7 Production in *Escherichia coli* B23 Is Not Inhibited By ΦX-174 at a Multiplicity of Infection of 10

Cordelia Cheng, Natasha Lee, Armin Mortazavi, and Caitlin Pritchard
Department Microbiology & Immunology, University of British Columbia

Understanding molecular interactions between bacteriophages in a co-infection is important for predicting progeny production which is used as an indicator of bacteriophage success in an infection ΦX-174 and T7 are *Escherichia coli* bacteriophages that both use lipopolysaccharide as a receptor for adsorption and entry. In this study, both T7 and ΦX-174 were shown to bind to *Escherichia coli* B23, however only T7 produced progeny in this host. Given this distinction, we asked if ΦX-174 at a multiplicity of infection of 10 is sufficient to inhibit T7 progeny production during sequential co-infection of *Escherichia coli* strain B23. Our data indicated that ΦX-174 bacteriophage at a multiplicity of infection of 10 is insufficient to inhibit T7 bacteriophage progeny production in *Escherichia coli* B23. This demonstrates that ΦX-174 does not interfere with T7 adsorption, even though they share the same host cell receptor.

Bacteriophages are obligate intracellular parasites that undergo their replication cycle in a susceptible host cell characterized by five distinct stages: reversible binding, irreversible binding, genome ejection, replication, and progeny release (1). Viral co-infections can differ from mono-infections due to molecular interactions at different stages of the respective viral life cycles (2, 3). The outcomes of viral co-infections can be difficult to predict and are not well understood at the molecular level. Chan et al. have investigated co-infection of T4 and T7 bacteriophage in *Escherichia coli* C600 strain (2). T4 was shown to dominate over T7 during co-infection. Brewster et al. observed differential bacterial lytic cycles as a result of co-infections with MS2 and either T7 or ΦX-174 bacteriophage in *E. coli* strain C3000 (3).

In this study, our aim was to investigate sequential infection of *E. coli* with ΦX-174 and T7. Bacteriophage T7, a lytic phage with a double-stranded DNA genome, uses its non-contractile tail and tail fibers to interact with the host receptor, lipopolysaccharide (LPS), a heterogeneous molecule (1, 4, 5, 6, 7). On the other hand, ΦX-174, with a single-stranded, linear DNA genome, utilizes 12 apical capsomeres protruding from 12 vertices of its icosahedral capsid for attachment to the host receptor, LPS (1, 2, 5). The presence of calcium has been shown to be necessary for ΦX-174 adsorption (6). Receptor sites for T7 and ΦX-174 are located in LPS basal core region, where the former requires heptose and glucose residues and latter requires a galactose residue at the core terminal (1).

We hypothesized that ΦX-174 would inhibit T7 progeny production in *E. coli* strain B23 by competing for LPS receptors. To test this hypothesis we assayed plaque production of T7 in *E. coli* strain B23 after pre-incubating the host with ΦX-174 at a multiplicity of infection (MOI) of 10. This sequential phage infection approach using a non-permissive host to one bacteriophage strain offers a method for studying co-infection with viruses. Our results showed that ΦX-174 was unable to interfere with T7 development due to insufficient binding competition.

MATERIALS AND METHODS

Strains and growth conditions. ΦX-174 and T7 stocks were amplified in *E. coli* C and *E. coli* B23, respectively. *E. coli* strains and bacteriophage stocks were obtained from the Microbiology 421 culture collection from the Department of Microbiology and Immunology, University of British Columbia. *E. coli* C strain (catalogue number: 124400) and ΦX-174 stock (catalog number: 124425) were originally obtained from North Carolina Biologicals, and the T7 stock was a Microbiology 421 laboratory stock created in 2008. *E. coli* cultures in logarithmic growth phase were prepared by 100-fold dilutions of overnight cultures into 50 ml of Luria Bertani (LB) broth (10 g tryptone, 5 g yeast extract, and 10 g sodium chloride per 1 litre of distilled water). Cultures were then infected with 50 µl of original bacteriophage stock. In the culture infected with ΦX-174, 2mM of $CaCl_2$ was added to the broth to allow for ΦX-174 adsorption. Infected cultures were incubated at 37ºC for 2.5 hours until a reduction in turbidity was observed. The cultures were then centrifuged at 16,000 x g for 15 minutes to remove cell debris. Supernatants were removed and filtered using 0.22 µm Sarstedt Filtopur S plus filters (reference number: 83.1826. 102) and 100 µl of chloroform was added to the resulting purified phage stocks.

Each phage stock was titered via plaque assays (described below) using *E. coli* host strains C and B23 for ΦX-174 and T7, respectively. The identity of T7 was confirmed by PCR amplification using T7- specific primers (described below) and ΦX-174 identity was confirmed by a negative plaque result using *E. coli* B23, a non-susceptible host for ΦX-174.

Plaque assays. Bacteriophage stocks and samples were enumerated and plaque morphologies were characterized using the double agar overlay method described by Kropinski et al. (8). To demonstrate host range and media requirements in addition to titer, both phage stocks were plated on *E. coli* B23 and *E. coli* C with and without 2mM $CaCl_2$. All plaque assays were performed in duplicate and incubated for either 8 or 16 hours at 37ºC before observation and enumeration.

PCR and agarose gel electrophoresis. The T7 bacteriophage stock was confirmed to be the correct bacteriophage using PCR amplification primer pair T7 Major Cap with the following sequences: T7 Major Cap F (5'- GGC TCT GAC TAA GGC TCG TG- 3') and T7 Major Cap R (5'- GAT AAT CTG GTC CGC TTG GA- 3') primers. This primer set was provided by Richard White III. Primer T7 Major Cap was expected to yield a 409 base pair product from the major capsid protein gene (gp10A) of T7 bacteriophage. Invitrogen Accuprime *Taq* DNA

Polymerase System (catalogue number 12339-016) was used with Accuprime PCR buffer I in a 20 µl reaction according to the corresponding instruction manual (9), using 7.5 µl of a 1 in 1.0 x 10^6 dilution of T7 bacteriophage stock. The reaction was done on a Bio-rad Gene Cycler thermocycler with an initial denaturation step at 94ºC for 8 minutes followed by 35 cycles of amplification: denaturation at 94ºC for 15 seconds, annealing at 60ºC for 30 seconds, and elongation at 68ºC for 1 minute. The resulting PCR product was resolved on a 2% agarose gel in 1 x TBE buffer (89 mM Tris Borate, 2 mM EDTA; pH 8.3) at 100 V for 90 minutes and visualized with ethidium bromide staining and ultraviolet light using an AlphaImager MultiImage Light Cabinet (Fisher Scientific).

Infection of *E. coli* B23 with ΦX-174 bacteriophage. ΦX-174 adsorption to *E. coli* B23 was examined by incubating ΦX-174 with *E. coli* B23 and then enumerating the unadsorbed phage using plaque assays. The number of unabsorbed bacteriophage (PFU) was compared to the number of unadsorbed bacteriophage (PFU) when an identical amount of ΦX-174 was incubated with *E. coli* C (a known host for ΦX-174) to compare adsorptions efficiency with infection of *E. coli* B23.

E. coli B23 and *E. coli* C cultures in logarithmic growth phase were prepared using 100-fold dilutions of overnight culture. Following this, 2 mM of $CaCl_2$ and 1.15 x 10^9 PFU/ml of ΦX-174 were added. The inoculated cultures were mixed for 5 minutes at room temperature and then centrifuged at 16, 000 x g for 5 minutes at 4ºC. The supernatant was removed and filtered using 0.22 µm Sarstedt Filtopur S plus filters (reference number: 83.1826. 102) to obtain unadsorbed phage and then 100 µl of each sample was plated in duplicate using the double agar overlay method (2 mM $CaCl_2$) on *E. coli* C. Control plates with no phage added were also made. After incubation for 16 hours at 37ºC, plaques were observed and enumerated.

Sequential infection of *E. coli* B23 with ΦX-174 and T7 bacteriophage. To test whether ΦX-174 adsorption inhibits T7 progeny production, *E. coli* B23 cells were infected with ΦX-174 at an MOI of 10 followed by T7 at an MOI of 1. *E. coli* B23 cells infected with T7 after the sequential infection was enumerated using a plaque assay. The observed plaque count was then compared to plaque assay results from cells that were inoculated with T7 only.

An overnight culture of *E. coli* B23 was diluted to obtain 1.0 x 10^6 CFU/mL for use in each binding condition. In the first condition, cells were inoculated with ΦX-174 at an MOI of 10 in the presence of 2 mM $CaCl_2$. After 5 minutes of mixing at room temperature, the inoculated cells were centrifuged at 16,000 x g for 5 minutes at 4ºC. The resulting supernatant was discarded and cells (with any adsorbed bacteriophage attached) were resuspended and inoculated with T7 bacteriophage at an MOI of 1. The T7-inoculated culture was mixed, spun, and resuspended as described above and then 10 µl of the resulting suspension was plated in duplicate using the double agar overlay method on *E. coli* B23 without $CaCl_2$.

In the second condition, cells were inoculated with T7 only at an MOI of 1 and in the third condition cells were inoculated only with ΦX-174 at an MOI of 10. Identical mixing, spinning, resuspension, and plating methods were used (as described above) with 2 mM of $CaCl_2$ added to steps involving ΦX-174 inoculation. Approximately 10 PFU of T7 bacteriophage stock was also plated in duplicate to confirm T7 plaque formation and control plates with no phage added were also included.

RESULTS

ΦX-174 was unable to produce progeny in *E. coli* strain B23. Differentiating bacteriophage infections is essential in this study in order to eliminate cross-contamination and to help identify different phage progeny in subsequent experimental steps. We discovered that T7 and ΦX-174 had similar plaque morphologies on *E. coli* C with presence of $CaCl_2$, but they are distinguishable on *E. coli* C without $CaCl_2$ or on *E. coli* B23 (Fig. 1). ΦX-174 did not form plaques when plated on *E. coli* B23 or on *E. coli* C without $CaCl_2$ while T7 produced plaques in all plating conditions (Fig. 1). Although plaque morphology was expected to be homogenous for each type of bacteriophage growing under identical condition, a wide range of clearing and halo diameters were observed for both bacteriophages (Fig. 1). Generally, the halos of the T7 plaques produced on *E. coli* B23 were 1.5 times larger than those produced on *E. coli* C (Fig. 1). Additionally, when propagated with $CaCl_2$, T7 plaques had 2 to 5 times smaller clearing centers on *E. coli* B23 relative to those on *E. coli* C.

Potential contamination due to inconsistent plaque morphology was eliminated by PCR analysis of T7 stock. An expected product size of approximately 400 bp was observed after PCR amplification of T7 stock and subsequent visualization using agarose gel electrophoresis (results not shown). Together with differential plating assay performed on ΦX-174 stock indicated that both samples contained the correct bacteriophage type (6, 23). ΦX-174 stock was found to have no T7 contaminant since growth was observed on E. *coli* C (with $CaCl_2$ addition) but not on *E. coli* B23, a non-susceptible host for ΦX-174 (6, 11, 12). Taken together these data suggested that although plaque morphology was an inadequate marker for identifying T7 and ΦX-174 bacteriophages, E. *coli* C was an adequate host to distinguish T7 bacteriophage progenies from ΦX-174 progenies in subsequent tests to verify bacteriophage identity.

ΦX-174 adsorbed to *E. coli* B23. Knowing that ΦX-174 was not able to produce progeny in *E. coli* B23 (Fig. 1), we asked whether ΦX-174 was able to bind to the bacterial surface of this particular host. To address this question, we performed a binding assay that compared the adsorptive ability of ΦX-174 on *E. coli* B23, a non-permissive host, to that of on *E. coli* C, a permissive host (Fig. 1). Our results showed that average number of unadsorbed phage (11 PFU) was equal for both conditions using either *E. coli* B23 or *E. coli* C infected with same amount of ΦX-174. This plaque count was approximately 10-fold less than what would have been expected if ΦX-174 did not bind to cells at all. This indicated that ΦX-174 adsorption to *E. coli* B23 and to *E. coli* strain C was similar regardless of ΦX-174's ability to replicate. Using this data, we were able to test the impact of ΦX-174 binding interference on T7 progeny production by using *E. coli* B23.

ΦX-174 did not inhibit T7 plaque formation in *E. coli* B23. Based on the previously established result that ΦX-174 was only able to adsorb but not propagate in *E. coli* B23, T7 bacteriophage's ability to produce progenies after the cells have been infected with ΦX-174 at 10 MOI were performed on this strain. *E. coli* B23 was inoculated with

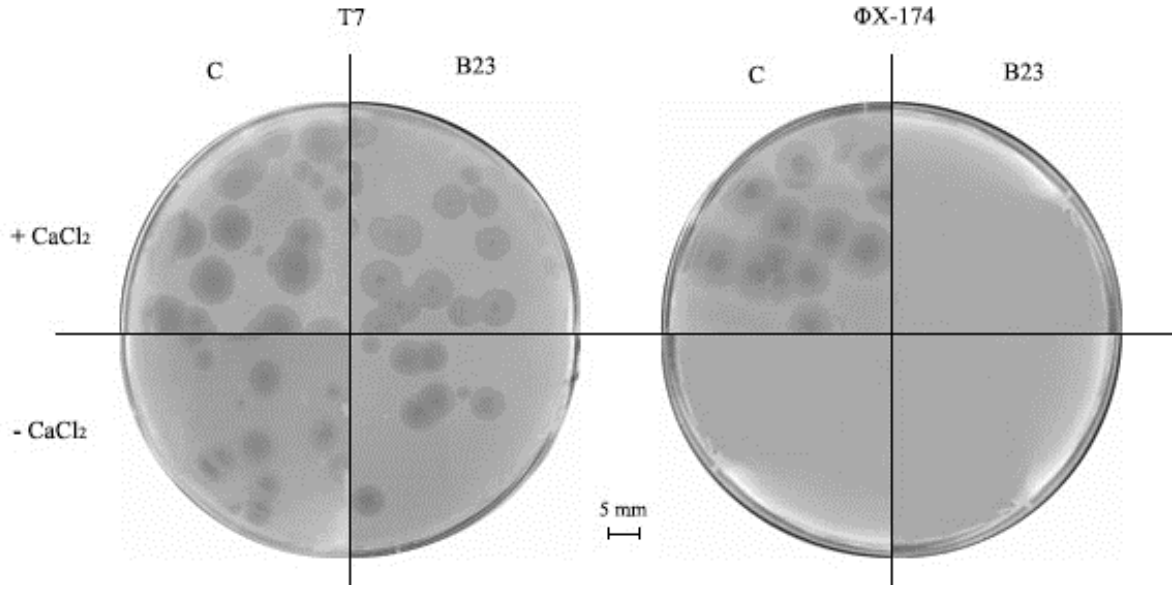

FIG 1 Plaque morphologies of T7 and ΦX-174 on *E. coli* strains C and B23, with and without 2 mM $CaCl_2$. Each quadrant represents a different plate.

ΦX-174 prior to the introduction of T7. The amount of T7 bacteriophage plaques resulting from adsorbed T7 phage after ΦX-174 pretreatment was very similar to the amount of T7 plaques after infecting the cells with T7 alone.

At an MOI of 1, T7 produced 287 PFU without ΦX-174 pretreatment, and 272 PFU after ΦX-174 pretreatment. The difference in PFU between the two binding conditions was within one order of magnitude, demonstrating that while both T7 and ΦX-174 required LPS for adsorption, ΦX-174 phage, at an MOI of 10, did not interfere with subsequent T7 infection of *E. coli* B23.

DISCUSSION

Molecular interactions between different bacteriophages in a viral co-infection determine the downstream progeny production of respective bacteriophages. From an evolutionary standpoint, limited host resources often encourage bacteriophage to implement interfering strategies to outcompete the other co-infecting bacteriophage. The interference can occur throughout the viral replication cycle. In this study, we aimed to investigate binding interference between bacteriophages attaching to closely-located host receptors in a co-infection. We used ΦX-174 and T7 as model bacteriophages since both of them bind to LPS. We also assumed that since *E. coli* B23 is a non-permissive host for ΦX-174, using this particular host would eliminate possible intracellular interferences from ΦX-174 to T7 development. However, our data was insufficient to confirm the absence of intracellular interference.

LPS consists of three regions: lipid A, core sugars, and the O-antigen (7). Notably, LPS is not always identical at a molecular and structural level across different strains of *E. coli* or even within the same strains (1, 7). This is termed LPS heterogeneity (6). In particular, the O-antigen varies with respect to different linkages made between monosaccharides and in its presence altogether (7).

E. coli C, a commonly-used host for ΦX-174, possesses several properties that allow phage adsorption and genome entry (5, 10). *E. coli* C has R1 type LPS core, one of the core sugar types sensitive to ΦX-174; however Michel et al. demonstrated that ΦX-174 is in fact able to bind to a variety of LPS morphologies (5). Therefore, our experimental finding on ΦX-174's ability to bind to both C and B23 was supported.

Plating analysis of ΦX-174 demonstrated permissiveness of *E. coli* C to ΦX-174 viral infection in the presence of $CaCl_2$. This requirement for $Ca2^+$ for ΦX-174 infection was consistent with previous literature (11, 6, 12). Host recognition of ΦX-174 is facilitated by the viral G and H surface proteins' adsorption to a specific region in the LPS core (12, 13). The amino acid residues in the proteins' recognition sites require $Ca2^+$ in order to obtain proper conformation for binding (11, 6, 12). Hence, $Ca2^+$ is required for ΦX-174 to infect its host.

Reversible and irreversible binding of ΦX-174 depends on the recognition of specific LPS structures by the G and

H proteins (6). While subsequent DNA injection into the host cell is partially facilitated by the G and H proteins, some studies suggest that this activity occurs directly through the inner membrane after viral binding to a second receptor located in the periplasm (6, 14). Although the exact identity is still unknown, several studies had proposed the existence of a second receptor

for ΦX-174 (6, 14, 15). Hence, the observed binding of ΦX-174 on *E. coli* B23 might have been due to the absence of this second receptor in *E. coli* B23.

An alternative explanation for ΦX-174's ability to adsorb to *E. coli* B23 but not replicate is the presence of a type I restriction-modification (R-M) system in this *E. coli* strain (16). The type I R-M system identifies non-host DNA based on its lack of methylation and cleaves it into small pieces (17). This effectively inactivates otherwise susceptible bacteriophage such as ΦX-174. Many types of bacteriophage, including T7, have developed resistance to this type of defense mechanism; however, ΦX-174 does not have any of these adaptations (18, 19). Therefore, even though ΦX-174 is able to adsorb to the surface of *E. coli* B23, its DNA may be rapidly degraded upon entry into the cell by cellular restriction enzymes. In contrast to *E. coli* B23, *E. coli* C has no restriction modification system, making it a susceptible host for ΦX-174 (16). This may explain how ΦX-174 is able to bind to both strains of *E. coli*, but only produce progeny in the *E. coli* strain C.

With ΦX-174 host range validated in our results, we were able to examine the effect of ΦX-174 binding to LPS on T7 bacteriophage's ability to subsequently bind and infect *E. coli* B23 while eliminating the possibility of ΦX-174 contamination in plaques. Furthermore, we also exploited ΦX-174's $Ca2^{+}$ requirement as a control for the occurrence of ΦX-174 binding where necessary.

Michel et al. found evidence that hosts sensitive to ΦX-174 infection all have rough LPS O-antigen, meaning that their O-antigen is missing and the core antigen is exposed (5). This suggests that rough O-antigen characteristic is a requirement for susceptibility to ΦX-174; indeed, *E. coli* C has this characteristic (5). Likewise, *E. coli* B23 also has this rough LPS phenotype since, like ΦX-174, T7 requires rough LPS for binding (1), and we successfully demonstrated T7's ability to infect this host. According to Michel et al., however, lack of an LPS O -antigen appeared to be necessary but not sufficient for ΦX-174 infection.

The attachment of ΦX-174 could have been reversible, which may have contributed to subsequent displacements of ΦX-174 by T7. The reversibility of ΦX-174 adsorbing to its receptor is affected by temperature, pH, and $CaCl_2$ concentration (20). It has been shown by Fujimura and Kaesberg that the optimal attachment temperature for ΦX-174 is 36°C with 0.1 M $CaCl_2$ (20). They demonstrated that the number of unbound phage - essentially the degree of reversible binding - increased above and below this temperature. Our mixing process was performed at room temperature. Therefore, it is possible that the ΦX-174 phage bound, then unbound - or never bound at all, allowing the T7 to adsorb without any competition. Fujimura et al. also suggested a minimum $CaCl_2$ concentration of 10 mM (20), whereas our experiment only used 2 mM following the instruction manual of Kropinski et al. (8). The 10 mM suggestion is may be outdated, but it is still possible that a higher concentration of $CaCl_2$ may allow for a more irreversible attachment of ΦX-174 to its receptor.

It is also possible that a MOI of 10 may be too low even with irreversible attachment of ΦX-174 to the receptors. Calculations using the average surface area of *E.coli* B23 and the average diameter of the ΦX -174 virion reveal that, if ΦX -174 bound to every available space on the host cell, an MOI of 33,263 would be needed to reach a saturating MOI (21, 22). Although this value is likely a gross overestimate of the true saturating MOI, at an MOI of 10, T7 may have been able to adsorb without any interference as there may have been many available receptors unbound by ΦX-174. It would be ideal to repeat this experiment with an MOI that is known to saturate all receptors. This leaves open the possibility that competition can occur but that we did not optimize the conditions for it.

In conclusion, ΦX -174 bacteriophage did not inhibit T7 plaque production in *E. coli* B23 after sequential infection. This result indicates that ΦX -174 does not interfere with any stage of the T7 replication cycle although they share the same receptor. This demonstrates that viruses which share the same receptor but utilize different parts of the molecule may not compete with each other during co-infection for binding sites or other cellular resources.

FUTURE DIRECTIONS

To determine whether ΦX-174 pretreatment saturates all available host LPS receptors irreversibly, a series of binding assays with increasing MOIs of ΦX-174 on *E. coli* B23 under optimized conditions should be performed. Based on previous research, optimal conditions would use 36ºC, and a higher CaCl2 concentration than what was used in this study (20). Additional assessment could be employed to confirm the sensitivity of ΦX-174 adsorption with respect to temperature and CaCl2 concentration. For testing the proposed optimal binding condition for ΦX-174, all factors should be kept constant while either temperature or CaCl2 concentration is varied. Enumeration of the unbound bacteriophage would determine the reversibility of ΦX-174 binding under the different conditions.

To determine the MOI that would achieve host receptor saturation, ΦX-174 should be incubated with *E. coli* B23 with a range of increasing MOI values. After sufficient time has passed for ΦX-174 to bind to *E. coli* B23, unbound bacteriophage could be separated from bound bacteriophage via centrifugation and enumerated by plaque assay using *E. coli* C as the host. The ideal MOI will be the approximate value at which the number of unadsorbed PFUs begins to increase sharply, indicating that a saturation point has been reached. If ΦX-174 concentration is too high, it will induce host cell lysis that is not caused

by lytic viral infection, known as "lysis from without" (23). To avoid this outcome, it is necessary to obtain an MOI that reflects the highest number of unbound bacteriophage possible that will saturate all receptors without causing host cell lysis.

To further investigate the inability of ΦX-174 to produce progeny in *E. coli* B23 in spite of the ability to adsorb, future studies could explore whether ΦX-174 genome is successfully injected into the host. In order to see whether ΦX-174 is able to inject its genome into the host, the host type I R-M system could be inhibited or removed via knock-out mutation and the resulting strain could be tested for ΦX-174 susceptibility. Alternatively, the ΦX-174 genome could be methylated by introducing a methylase that recognizes and methylates the ΦX-174 genome to protect ΦX-174 from the host's restriction system. Host susceptibility to methylated ΦX-174 would indicate that the host restriction system is responsible for inhibiting ΦX-174 progeny production. Either of these approaches could also be used to prevent cleavage of ΦX-174 DNA by host restriction enzymes that allow the use of PCR to detect presence of full ΦX-174 genome in the host cell.

Lastly, our experiment can be further applied to study binding interactions between other phages that share similar host viral receptors such as T4 and T3 bacteriophages, which use the same region of LPS as a receptor (24). If the host selected supports infection by both bacteriophages, generation of ghost particles, virion particles without a genome, can be used to saturate the host receptors at the pretreatment stage in order to avoid unwanted viral replication and progeny production (25). Ghost particles can be prepared by treating the bacteriophage of interest with lithium chloride or subjecting them to freeze-thawing cycles (25). Alternatively, sodium azide, which stops host cell replication in a reversible fashion, could be used to synchronize viral infections to prevent premature release of viral progeny (26).

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