

Co-infection of C3000 *Escherichia coli* with Bacteriophages MS2 and, T7 or ΦX-174 Results in Differential Cell Lysis Patterns

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Bacteriophages can be used as a model for studying the dynamics of viral co-infection in a susceptible host. *Escherichia coli* strain C3000 was chosen as a common, susceptible host to determine the effect of competition on the lifecycle of MS2 co-infected with ΦX-174 or T7. Strain C3000 expresses lipopolysaccharide, necessary for attachment of ΦX-174 and T7, and male sex pili necessary for the attachment of MS2. The C3000 strain was infected with MS2 and ΦX-174 or with MS2 and T7 at a multiplicity of infection (MOI) of 1 or 2. In general, MS2 co-infection with ΦX-174 or T7 resulted in an earlier incidence of lysis compared to single infection with MS2 or ΦX-174 alone. Also, decreased lytic time was greatest at a higher MOI in MS2 co-infection with ΦX-174 but this trend was reversed in MS2 co-infection with T7. In this case, decreased lytic time was greatest at the lower MOI. This indicated that the intracellular interactions of one phage affected the lifecycle of the other. The effect of MOI on lytic time was inconsistent across strains and varied between ΦX-174 and T7.

It has long been established that two different bacteriophages are capable of infecting the same host (5). In 1970 such a case was reported by Ling *et al.* where they observed the simultaneous co-infection of Q-13 *Escherichia coli* with two RNA viruses, MS2 and Qβ (7). The results of the study by Ling *et al.* suggested that even though the progeny consisted of both competent and distinct MS2 and Qβ particles, productivity of infection was markedly lower, indicating competition between the phages likely occurred (7). In order to see if this effect is consistent across different infection models, *E. coli* strain C3000 was simultaneously infected with MS2 and T7 (MS2/T7) or MS2 and ΦX-174 (MS2/ΦX-174). These alternate bacteriophages differ from Qβ in their receptor requirement, to eliminate extracellular competition, and genome type, to test the impact of different genomes.

Loosely resembling poliovirus in size and structure, MS2 is a member of the family *Leviviridae* (2). It has a single stranded, positive-sense, RNA genome 3.6 kbp in size, and codes for four proteins (2). MS2 exclusively infects male *E. coli*; the presence and expression of the F plasmid produces pili, which MS2 uses for viral attachment (6). By comparison, ΦX-174 is a member of the *Microviridae* family (2). The linear, single stranded, 5.4 kbp DNA genome is encapsulated by a 25 nm shell and codes for 11 proteins. ΦX-174 uses lipopolysaccharide (LPS) as a receptor for host entry (2). T7 also uses LPS as a receptor and contains a DNA genome (2). However, the T7 genome is double stranded, linear, and 40 kbp in size (2). Consistent with

the T-type phage morphology, it exhibits the typical head, tail and leg physiology, although the tail is short and non-contractive. Strain C3000 is a derivative of K-12 *E. coli*, which expresses the F plasmid required for MS2 susceptibility (6).

The purpose of our study was to observe the impact of internal competition between bacteriophages on their replicative fitness in strain C3000 which may lead to establishing the possibility of developing a model for human pathogenic co-infections. As we observed that all three phages produce a lytic infection, it was possible to track their replication by observing the growth of strain C3000. In doing so, we posed the experimental question: do bacteriophage co-infections alter the growth of strain C3000 in comparison to single bacteriophage co-infections? We hypothesized that competition between bacteriophages would increase the time between phage adsorption and strain C3000 lysis. In other words, we predicted that co-infection would slow the intracellular phage life cycle, thereby increasing the time until cell lysis. The results of our study were able to show that not only are MS2, ΦX-174 and T7 able to infect strain C3000, but also that competition can alter the replicative cycle of each phage during co-infection.

MATERIALS AND METHODS

Strains and reagents. *E. coli* strain C3000, bacteriophage MS2 and ΦX-174 strains were obtained from the C. Suttle Lab, Department of Microbiology and Immunology (MBIM) at the University of British Columbia (UBC). The T7 strain was obtained

from the Microbiology 421 Laboratory stock from the Department of MBIM at UBC. Bacterial cells were grown in Luria-broth (LB) (0.5% w/v Bacto Yeast Extract, 1% w/v Bacto Tryptone, 0.5% w/v NaCl, pH 7). Soft-overlay plates were prepared with LB agar (0.7% and 1.5%). 10X phage co-factor buffer was added to phage-C3000 infections to provide the necessary cofactors (2.41% w/v Tris-HCl, 5.85% w/v NaCl, 1.2% w/v MgSO₄, 1.11% w/v CaCl₂, pH 7.4).

Amplification of acquired phage stocks. Amplification of the phage stocks were performed by adding 3 ml of LB, 500 µl of strain C3000, and 150 µl of infectious phage stock. Amplifications were incubated aerobically overnight at 37°C. Amplification lysates were centrifuged for 2.5 minutes at 8,500 rpm, filtered (through a 0.22 µm Millipore HAWP filter), and stored at 4°C. Plaque assays were used to quantify the stock solutions as described below. Amplifications were repeated until phage titres were above 10⁸ pfu/ml.

Plaque assays of MS2, ΦX-174 and T7 phage using C3000.

The phage were prepared and quantified by the soft-agar overlay (double-agar layer) method of plaque assay using *E. coli* strain C3000 as the host. The C3000 strain was grown in 1X LB media. The LB media for the top and bottom layers used in the plaque assays contained 0.7% and 1.5% agar, respectively. The Petri dishes containing the bottom layer were warmed to 37°C. The overlay was melted to a liquid state and maintained at 48°C until plating. 50 µl of 10X phage co-factor buffer, 150 µl of strain C3000 in exponential at early stationary phase, and 170 µl of phage were added to 3 ml of 0.7% overlay prior to plating. The inoculated overlay was then poured onto solidified 1.5% LB agar plates. The inoculated plates were incubated aerobically overnight at 37°C. The phenotype and number of plaques were then observed. Plaque counts were used to titre phage concentration.

Growth curve generation of the C3000 strain singly or co-infected with MS2, ΦX-174 or T7. Strain C3000 was grown overnight in LB at 37°C with aeration and was diluted to an OD₆₀₀ of 0.2 to 0.4. For single infections, cells were added to a 2 ml cuvette, along with MS2, ΦX-174 or T7 at a multiplicity of infection (MOI) of 1 and 2. For co-infections, treatments of MS2/ΦX-174 and MS2/T7 were added to cells at a combined MOI of either 1 or 2. Turbidity was measured using an Ultrospec 3000 ® at 5 minute intervals until turbidity readings remained constant.

RESULTS

MS2, ΦX-174 and T7 phage are capable of infecting strain C3000 resulting in cell lysis over time. The phages were tested for their ability to infect strain C3000 using plaque assays and phage amplifications. All three phage were capable of lysing the host and producing plaques of about 5 mm in diameter. An example of MS2 phage growth can be observed in Figure 1. Infectivity was also observed in the phage amplifications, as culture turbidity rapidly decreased within four to six hours. Clearing of the C3000 strain in log growth was observed only in infected cell cultures and not in uninfected cell cultures, indicating cell lysis had occurred due to infection. A decrease in culture turbidity represented increasing cell lysis.

Strain C3000 grown in LB was infected with either T7, ΦX-174 or MS2 phages at an MOI of 1 and 2. All three phage were able to cause cell lysis over time at both MOI of 1 and 2 (Fig. 2). The resulting growth curves, depicted in Figure 2, showed similar, increasing rates of growth of strain C3000 up until a time point of one hour. After approximately one hour, cells infected

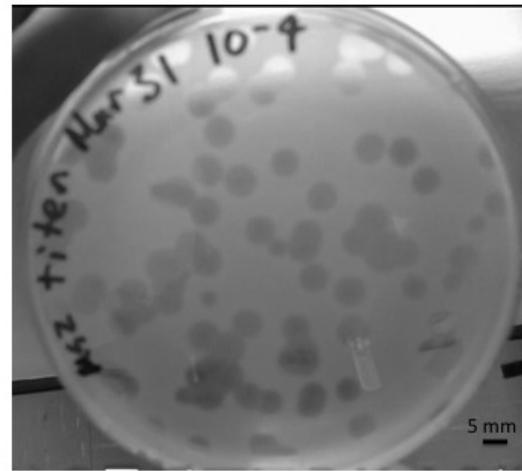


FIG. 1. Plaque forming units of MS2 phage on a lawn of *E. coli* strain C3000.

with T7 began to lyse, followed by ΦX-174, then MS2. Decreasing turbidity was observed for about 40 minutes to one hour after the initial decline began. In general, cells infected with phage at an MOI of 2 experienced a decline in turbidity earlier than cells infected an MOI of 1. Cell lysis, which was measured by decreasing turbidity, continued progressively until infected cultures were no longer turbid. The drop in turbidity of cells infected with T7 and ΦX-174 eventually reached a plateau, which indicates low and unchanging cell concentration. Cells infected with MS2 did not reach an observable plateau within the time constraints used in this experiment, however, a drop in turbidity was observed (Fig. 2).

Co-infection of strain C3000 with either MS2/T7 or MS2/ΦX-174 results in different patterns of cell lysis compared to single phage infections. Strain C3000 grown in Luria-broth was co-infected with either MS2/T7 or MS2/ΦX-174 at an MOI of 1 and 2. Similar to what was observed in the growth curves during the single infections, an initial rise in turbidity was observed, followed by a decrease in turbidity and an eventual plateau (Fig. 3). This result indicates that strain C3000 continued to grow during the initial infection, however, started to lyse as the infection proceeded. However, cell lysis began much sooner in co-infected cells compared to singly infected cells. In co-infected cells turbidity decreased after 20 to 40 minutes whereas in the single-infected cells, a decrease in turbidity was observed after one hour. Additionally, in cells co-infected with MS2/ΦX-174, cell lysis began 20 minutes earlier compared to cells co-infected with MS2/T7 (Fig. 3). Interestingly, with MS2/ΦX-174, infection at a combined MOI of 2 resulted in earlier cell lysis compared to an MOI of 1. Conversely, infection

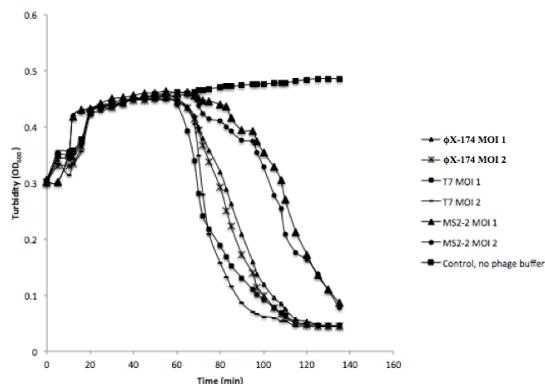


FIG. 2. The effect of MS2, T7, or Φ X-174 infection on the growth of C3000.

with MS2/T7 at an MOI 1 lysed cells earlier than infection with MS2/T7 at an MOI 2.

DISCUSSION

Growth curves of C3000 infected with either MS2, Φ X-174, or T7 had a similar growth curve pattern, but the point at which cell lysis began varied between infections. The difference in time to cell lysis in each infection could be due to two causes. First, this result may indicate that each phage causes lysis at different rates, with T7 being the fastest and MS2 being the slowest. This could be due to the speed at which the phage attaches to the host receptor, enters the cell, replicates, assembles or lyses the cell (13). If this is the case, T7 appears to be able to produce viral progeny, resulting in cell lysis, more quickly than the other two phages. Importantly, when phage were added to the host at an MOI of 2, lysis was observed sooner than when phage were added at an MOI of 1. This observation could be due to increased stress on the host, resulting in lysis. Alternatively, calculations of phage titres were not preformed in duplicate and may have been inaccurate between the different phages, which could result in more T7 added than MS2 during individual infections.

The comparison between the co-infection and single infected strain C3000 growth curves yielded an unexpected result which is contrary to the findings of Ling et al. and our hypothesis that phage co-infection would result in competitive inhibition (7). In the study by Ling et al. they found that co-infection with MS-2 and Q β decreased the productivity and efficiency of infection (7). This could be attributed to external competition; both Q β and MS2 employ the same host receptor (7). With respect to our study, MS2 does not share the same receptor as either T7 or Φ X-174, which both use LPS (2). As such, it is possible that an

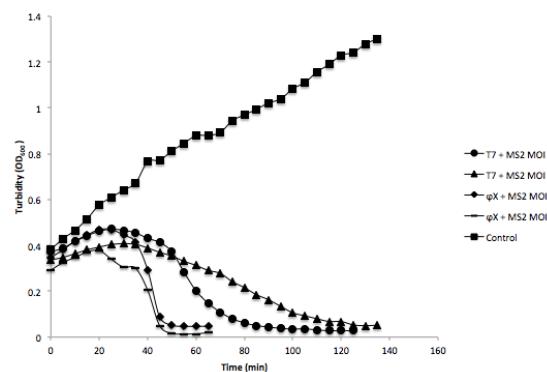


FIG. 3. Lysis of C3000 during co-infection with MS2/T7 or MS2/ Φ X-174 at variable MOIs.

intracellular synergistic relationship between Q β and MS2 may have existed, but was overshadowed by competition for entry-receptor binding sites. If this factor was removed, perhaps Q β and MS2 co-infection would have demonstrated kinetics similar to our observations.

While it is not possible to confirm the number of competent progeny of both phages for each co-infection treatment, the co-infection growth curves suggest the two phages are interacting either indirectly or directly such that the length of their life cycle is affected. Assuming the three tested phages are capable of adequately partitioning host resources and pathways, an obvious explanation is that a more serious infection, leading to more immediate C3000 lysis, is produced (6). A study by Jain *et al.* has demonstrated that MS2 diverts all host cell metabolic resources to the synthesis of amino acids and upregulates the pentose phosphate pathway, instead of the production of biomass as in the uninfected cells (6). This demonstrates that MS2 is able to hijack host cell pathways for its own production of viral proteins. It is possible that MS2 may dysregulate certain host cell pathways that play a major role in the lifecycle of another phage, leading to a beneficial or detrimental effect. For example, MS2 upregulation of amino acid synthesis may be more efficient than Φ X-174, and thus better provide amino acids for Φ X-174 replication in a co-infection compared to a single infection. The likelihood of such a synergistic interaction occurring in a co-infection seems overshadowed by likely competition for host cell resources, such as ATP or ribosomes. However, a significant portion of the MS2 life cycle does not compete with either T7 or Φ X-17 because MS2 replication does not require a DNA polymerase nor dNTPs and their associated components. So in this case, there should be less competition for resources involving genome replication. Since a significant portion of the MS2 life cycle does not compete with either T7 or Φ X-

174; replication does not require a DNA polymerase nor dNTPs and their associated components, then intracellular competition for resources should be reduced. This explanation is further supported by the Hepatitis B virus (HBV) and Hepatitis C virus (HCV) pathogenesis co-infection model. Co-infection with HBV and HCV is known to produce a more severe infection in humans, linked to an increase in viral replication within the body (9). This demonstrates how viral co-infection can enhance viral fitness. An alternative explanation is that one phage produces allelopathic products to enhance and facilitate the replication of the other co-infected phages (1). While the in-depth host determinants and interactions with each of MS2, Φ X-174, and T7 have yet to be elucidated, given other examples in nature such as the synergistic relationship between certain viruses and bacteria that is related to the production of type-III pneumonia (10) we believe it is possible that co-infection leads to a more severe infection as a result of synergistic relationship between the phages.

More likely, this observation could be due to an increased efficiency of adsorption between strain C3000 and the phage because of the higher concentration of phage added in MOI 2. Phage adsorption rate is a function, among other variables, of the product of the concentrations of bacteria and phage (4). Higher rates of adsorption could promote viral uptake and thus increase the speed at which infection occurs (3).

In co-infection with MS2/T7 a higher MOI was correlated with a longer time observed until cell lysis began, compared to cells infected at a lower MOI. Although this contrasts the observed effect in the MS2/ Φ X-174 co-infection, it could be seen to agree with the findings of Ling *et al.* (7). The likelihood of cells being infected with MS2 and T7 at a combined MOI of 2 is higher than when infected at an MOI of 1 (3). Therefore, the formal experimental condition more accurately represents the co-infection model that we wished to study. Therefore, a longer time until lysis in the MOI 2 infection could indicate competitive inhibition between T7 and MS2 similar to that reported between MS2 and Q β (7). Competitive inhibition between T7 and MS2 could arise from mutual requirements for cellular macromolecules such as nucleotides, amino acids, and ribosomes (8).

There is a difference between T7 and Φ X-174 in infected cells that depends on MOI in MS2 co-infection. This indicates that there could be interactions between phages during co-infection that can vary depending on the species. It is reasonable to conclude that T7 and Φ X-174 had opposite interactions with MS2 because there are significant genotypic differences between these two phages. T7 contains a very large (40 kbp), double-stranded, linear genome whereas the Φ X-

174 genome is much smaller (5.4 kbp), single-stranded, and circular (2). T7 has a 15 times larger genome size than the Φ X-174 genome, which suggests that replication of T7 may take longer.

Alternatively, it is possible that MS2/ Φ X-174 co-infection would have resulted in competitive inhibition similar to what was observed with MS2/T7, but inaccurate titering of MS2/ Φ X-174 resulted in a low MOI. There may not have been enough cells co-infected with MS2/ Φ X-174 to produce an observable effect.

In summary, strain C3000 has proven to be a usable model for the study of phage co-infections with MS2, Φ X-174, and T7; we have confirmed it is susceptible to MS2, Φ X-174, and T7. These phages each progress through their life cycle at different rates and induce cell lysis at different times. Co-infecting strain C3000 with MS2, and T7 or Φ X-174 shortens the time it takes for phages to progress through their life cycle compared to single infection. However, the MOI of co-infection has an opposite effect on this observation between T7 and Φ X-174. This suggests that co-infection causes earlier cell lysis but the cause of this effect is dependent on the specific interactions between the phage strains in context.

FUTURE DIRECTIONS

To ensure the validity and accuracy of our results, the experimentation protocol needs to be repeated with precise viral stock titres.

To determine if the phage genome type can be related to co-infection intracellular competition, it would be beneficial to quantify the phage progeny from the filtered lysates of the two co-infection treatments. In light of this, we propose the use of qPCR (or RT-qPCR for MS2) to provide both a more accurate and more efficient method of quantifying phage progeny. Alternatively, the use of a non-F+ plasmid E. coli strain (thus lacking the MS2 receptor) such as DH5 α could be employed to distinguish between MS2 and Φ X-174 or T7 (11). Filtrates would be plated concurrently on strain C3000 and strain DH5 α ; the difference in plaque forming units would be equivalent to MS2 progeny produced from the co-infection (6).

Finally, to acquire a better picture of the significance of genome type with respect to intracellular co-infection competition, we believe two additional families of bacteriophages should be tested against an MS2 infection, to further observe the effects of genotype on co-infection. These proposed bacteriophages should include a member of the double stranded RNA *Cystoviridae* family and of the double stranded, circular DNA *Inoviridae* family. These phage could provide more information on the effects of replication methods during co-infection. Similarly,

strain C3000 could be co-infected with T7 and ΦX-174, which both share LPS as a receptor, and would provide more information on the effect of receptor competition.

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