Journal of Experimental Microbiology and Immunology (JEMI) Vol. 18: 156 – 161

Co-inoculation of *Escherichia coli* B23 by T4 and T7 bacteriophages results in competition shown by an overall drop in phage progeny

Khue-Tu Nguyen, Karen Simmons, Igor Tatarnikov
Department Microbiology & Immunology, University of British Columbia

Co-inoculation and co-infection are phenomena that occur naturally in the environment that have implications on. In order to explore competition between T4 and T7 phages, one-step assays were carried out on T4 and T7 mono-infected samples, and a sample co-inoculated by both T4 and T7. Mono-infections were performed at an MOI of 5 in order to ensure infection, and the co-inoculation had an MOI of 5 for both T4 and T7 resulting in a total MOI of 10. It was found that at 90 min post co-inoculation, the overall titer of both phages decreased with respect to their mono-infected controls, 97% for T4 and 98% for T7. This suggested that actual co-infection could have occurred and competition was carried out within the host cell. This effect demonstrated that even though there seems to be interference between the two phages in co-inoculation, neither phage out-competes the other.

T4 and T7 are well-studied bacteriophage capable of infecting *Escherichia coli* (*E. coli*) (1, 2). They contain double-stranded, linear, DNA genomes, with T4 having approximately 169 kbp coding for around 300 genes (1,3) and T7 having roughly 40 kbp and approximately 56 genes (2). There is potential for these phage to compete with one another for the same host cells. As such, they can be used as a model for investigating co-inoculation, co-infection and competition, which are phenomena that occur naturally in both medically and environmentally relevant infections (4, 5).

Competition between phage has been previously observed (6, 7). There are several mechanisms by which phage have the potential to compete with one another in a natural environment. Both T4 and T7 have endonucleases that serve to breakdown host DNA into nucleotides for use by the phage for replication (1,2). In T4, these enzymes have been shown to also breakdown competing phage DNA in a co-infection (3). These phage can also protect their DNA from self-degradation by their own endonucleases and potentially competing endonucleases by modifying their DNA (2, 3). T4 has also evolved a mechanism which causes DNA from competing phage to be trapped in the periplasm if it is injected after another T4 phage has already established an infection in that host (3).

The differential plaque morphologies of T4 and T7 when infecting *E. coli* B can be used to determine the titer of each phage during co-inoculation. T4 plaques are small, point-like plaques whereas T7 has larger plaques with halos. Using this, the competition of T4 and T7 in a co-inoculated culture of *E. coli* B23 was investigated to determine whether these phage interact during the infections arising from co-inoculations. , with specific comparison to the mono-infection controls, measured by the concentration of viral progeny produced by each phage in a one-step assay.

MATERIALS AND METHODS

Strains used in this study. *E. coli* B23, *E. coli* C, *E. coli* C600, bacteriophages T4, T7, and ΦX-174 strains were all obtained from the Microbiology 421 culture collection from the department of Microbiology and Immunology, University of British Columbia.

PCR. PCR was performed to determine the purity of the bacteriophage stocks and plaques in this study. A total volume of 20 ul in each PCR reaction tube contained 1X AccuPrime™ PCR Buffer II (Life Technologies Inc.), 0.25 uM of each forward and reverse primers and 0.25 U of AccuPrime™ *Taq* DNA Polymerase (Life Technologies Inc.), 1 ul of stocks for template DNA or scraped overlay agar in location of plaque and sterile water to have a 20 ul reaction volume. A Biometra® TGRADIENT was run at 95°C for 5 min to activate hot start polymerase and to release DNA from phage particles. This was followed by 40 cycles of 95°C, 53°C, and 72°C each for 1 min. A 5 min extension time at 72°C was followed by a 4°C hold until the samples were run on an agarose gel. A 1% agarose gel was made using Ultapure™ agarose (Invitrogen) and Tris-borate-EDTA (TBE) buffer with 1 ul of SYBR® Safe DNA Gel Stain (Life Technologies Inc.). Imaging was done on a BIORAD ChemiDoc™.

Double agar overlay plaque assays. Procedure was followed as previously described (8). Briefly, 15% Luria agar containing 10 g tryptone, 5 g yeast extract and 10 g NaCl was use for the underlay petri plate and 7% Luria agar of the same composition for the overlay. At time of plating, 100 ul of *E. coli* host strain and 100 ul of desired dilution of phage was mixed gently into the 48 °C overlay agar and poured over the underlay agar plate. The plates were incubated at 37°C overnight before counting the plaques. All overnight growth of host bacteria and dilution were done using Luria broth (LB) with the same composition as the plates and overlay without the agar. When growing or titering ΦX-174 in *E. coli* C the overnight culture and inoculated phage were each supplemented with 10 mM $CaCl_2$.

T4 antibody neutralization. To examine purity of the T4 stock an antibody neutralization assay was performed. Stock T4 phage was diluted to a final plated dilution which resulted in 100-200 plaques in a final volume of 250 ul. The diluted stock T4 was mixed with 10 ul of T4 antiserum (Carolina Biological Supply Company, 12-4547) for 10 minutes at room temperature, or 10 ul of LB. Following the incubation the double agar overlay plaque assay was performed in duplicate.

Binding assay. To determine the host binding specificity of T4 bacteriophage, 10^8 *E. coli* B23, *E. coli* C600 and *E. coli* C cells were infected with T4 at an MOI of 0.02. Bacterial cells were treated with 1 mM sodium azide (NaN_3) to allow adsorption but

prevent replication (9). Samples were taken at times 0, 2.5, 5 and 15 min and diluted by a factor of 100 and spun at 8,000 × g for 1 min before plating the supernatant for the plaque assay. Total plated dilutions of 10^{-3}, and 10^{-4} were done in duplicate at each time point using *E. coli* B23 as the overlay host bacteria. Plates were incubated for 24 hr at 37°C and plaques counted to determine the difference in viral titer between time points.

Co-inoculation by one-step growth assay. Adapted from Stent (10). The term co-inoculation refers to the condition of including both phage in the initial infection, but without the assumption that individual host cells are actually infected by both phage at one time. Co-infection is the condition of one host cell being infected with both phage at the same time, whereas a mono-infection is when only one phage type is present in the infection. The turbidity of *E. coli* B23 cells was measured by spectrophotometer to obtain 10^8 cells. These cells were spun down, resuspended in sterile LB, treated with NaN_3 and infected with T4, T7 or both at an MOI of 5 for 5 or 20 min. The cells were then washed by pelleting, discarding the supernatant, resuspending in sterile LB with NaN_3, and repeating this a second time without NaN_3. This suspension was immediately diluted by a factor of 100. These diluted cells were considered to be the infected cultures which were sampled at times 0, 10, 20, 30, 40, 50 and 60 min. For time 0 and 10 samples, further dilutions were done to 10^{-4} and 10^{-5} and used for the previously described double agar overlay plaque assays. For the remaining time points, 10^{-5}, 10^{-6} and 10^{-7} were plated. These platings were all done in duplicate for the mono-inoculation. Time points 20-60 min were done in triplicate for the co-inoculation sample. When the experiment was repeated the time points were changed to 0, 60 and 90 min.

RESULTS

Purification of bacteriophage stock. T4 was expected to have small point-like plaques (11), and T7 larger plaques with halos (3), but T4 and T7 both appeared to have T7 plaque morphology when grown in *E. coli* C. They were then both grown in *E. coli* B23 and plated on *E. coli* B23 and *E. coli* C to determine if the large T4 plaque size was due to host strain variability. T4 did not grow in *E. coli* C and grew in the expected point-like plaques on *E. coli* B23, whereas T7 plaques looked the same regardless of the host strain. The small T4 plaques on *E. coli* B23 were then used to further propagate the phage. The T7 bacteriophage stock was purified by propagating it first in *E. coli* C, which eliminated T4, followed by propagation in *E. coli* B23 which eliminated any ΦX-174 contamination. The presence of T7 and ΦX174 contamination in the original T4 and T7 stocks and the purity of the working phage stocks were shown through PCR using primers listed in Table 1 for ΦX174, T4 and T7 (Fig 1).

Confirming plaque morphology and identity. The purification of stocks of T4 and T7 stocks along with the two plaque morphologies observed on *E. coli* B23 was confirmed by PCR with primers listed in Table 1. The PCR showed that the re-isolated stocks were free of contamination and that the plaque morphologies matched expectations, the point-like small plaque being T4 and large plaques being T7 (Fig 2). The purified T4 isolate was neutralized by anti-T4 sera, further confirming the purity. This result allowed quick and accurate identification of T4 and T7 purely based on plaque morphology. Due to the presence of a large opaque halo around the T7 plaques, T4 plaques were easily distinguishable within this halo, removing the confounding possibility of T4 plaques being lost within T7 plaques (data not shown).

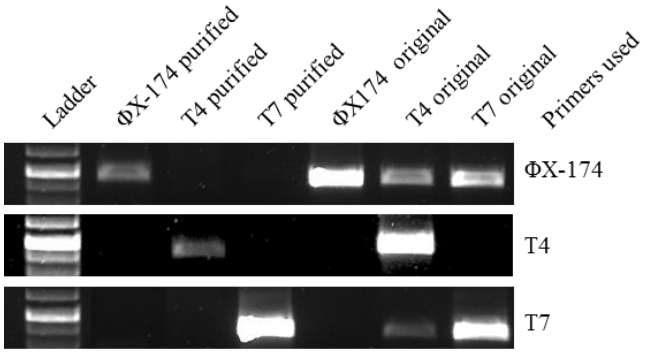


FIG 1 PCR could identify contamination in phage stocks. PCR was done on the original and purified stocks using the specified primers as per Table 1. Each picture corresponds to a different gel. The bright band in each ladder corresponds to the 500 bp band in theThermo Scientific 1kb Gene Ruler plus molecular ladder.

Binding assay to determine strain binding specificity. To examine the apparent inability of T4 to infect different strains of *E. coli*, a binding assay was performed. *E. coli* C showed no adsorption of T4 over a time period of 12.5 min, whereas *E. coli* C600 and *E. coli* B23 did. T4 adsorption into *E. coli* C600 was lower than *E. coli* B23 adsorption by 17% (Fig 3). According to these results, T4 is unable to bind to *E. coli* C, while retaining the ability to do so with *E. coli* B23 and to a lesser extent *E. coli* C600. The result for T4 and T7 was as expected; it is known that T4 binds to the OmpC in *E. coli* K-12 and lipopolysaccharide (LPS) in *E. coli* B23 (1). The result for *E. coli* C suggested that T4 is unable to use either of the known targets.

Co-inoculation for investigation of phage competition. At 0 min the infections were synchronised due to the presence of azide. This allowed both viruses to inject their DNA into the cells without interference from phage produced proteins. It was expected that by using a MOI of 5, the host cells would have been limiting at this point, only allowing for one burst and no further increase in titer; however this was not observed. The exponential growth in the mono-inoculated cultures continued past 60 min with at least one more burst occurring before 90 min (Fig 4). At 0 min into the time course experiment, the co-inoculation titers of T4 and T7 were reduced by 41% and 33% respectively compared to the mono-infection controls (Table 2). This change was not statistically significant but consistent across two separate experiments. This result suggested the possible occurrence of interference between T4 and T7 during the initial infection as compared to the mono-infection controls. Co-inoculation of T4 with T7 for 90 minutes in *E. coli* B23 resulted in a significantly decreased titer in both viruses as compared to mono-inoculation (Fig 4). At 90 minutes post co-inoculation, T4 titer was 97% lower and T7 titer was 98% lower than their respective mono-inoculation controls (Table 2). This result

TABLE 1 Primers used in this study.

Primer Name[a]	Sequence (5'-3')	Gene target	Product size (bp)
RW1_T7-majorcapR	GGCTCTGACTAAGGCTCGTG		
RW1_T7-majorcapF	GATAATCTGGTCCGCTTGGA	gp10A (T7 major capsid)	409
RW2_T4-majorcapR	ACCGATAACCCATGGAATGA		
RW2_T4-majorcapF	ATGCCGGTATCAACTGAAGC	gp23 (T4 major capsid)	452
RW3_phiXmajorCapR	CGCTCTAATCTCTGGGCATC		
RW3_phiXmajorCapF	ACGGACTGGAAACACTGGTC	gpF (φx174 major coat)	476

suggested that the co-inoculation had an effect on both T4 and T7 titer however neither out competed the other in this experiment. This was not as expected, since T4 was reported to outcompete T7 in a co-inoculation situation (7).

A consistent decrease in titer was observed between mono-inoculated cultures and co-inoculated cultures when compared separately or as a sum of the plaque counts in the mono-inoculated cultures (T4 and T7) to the total plaque count of co-inoculated plates (Table 2). This result was expected, as a similar decrease in titer due to co-inoculation of two viruses has been observed (12). The mono-inoculations provided the theoretical maximum number of plaques possible with the MOI used. The observed drop in the co-inoculation titer suggested that either the probability of infecting a cell was decreased in the co-inoculation or that one cell was infected by more than one type of virus and the combination interfered with normal phage development.

The main limitations in the results were the lack of culture replicates in the co-inoculation assay, and the binding assay. Subtle changes could have been observed to be statistically significant if there had been more samples to decrease the standard error. Another limitation of the co-inoculation assay was the lack of observable evidence for co-infection; the results neither supported nor refuted the possibility that two viruses simultaneously existed and competed in one cell, or simply competed for entry into the cell. Finally, the lack of CFU counts prevented assurance that all *E. coli* cells were lysed and therefore a limiting factor in the infection. Given these limitations, one can still conclude that T4 is unable to infect *E. coli* C and that there was a general effect on phage titer under co-inoculation conditions.

DISCUSSION

The decrease in titer observed at 90 minutes suggested actual co-infection occurred. The mono-inoculation control provided the maximal end of experimental titer and a decrease from that maximum can be attributed to fewer cells being infected due to more than one virus type infecting the same cell. Considering B to be the titer obtained from T4 mono-infection, and C to be the titer obtained from T7 mono-infection, and A being the titer of T4 and T7 obtained from the co-inoculation the formula B+C-A can be used to approximate the number of cells co-infected (6). According to this formula there is an increased number of co-infected cells as the titer drops (Table 2). This supports the theory of host cell not being limiting at 0 min but becoming so at 60-90 min thus forcing competition. However, the limitation of this model lies in its complete reliance on statistics rather than direct observation. Without visualizing both viruses infecting one cell it cannot be implied that the only explanations for the drop in titer is co-infection, as other factors may come into play.

It is possible that both T4 and T7 were able to bind to the same host cell and inject DNA and that competition was carried out intracellularly. This effect has been previously observed for the RNA bacteriophages Qβ and MS2 (6). Both T4 and T7 encode endonucleases that could be used in a competition-based setting. The T4 endonuclease II (Endoll) will degrade both host and competing phage DNA that does not have glucosylated hydroxymethyl cytosine (HMC) residues (3). However, the recognition sites for type II restriction enzymes, similar to Endoll of T4, are rare in the T7 genome (3). Although T7 also encodes an endonuclease, T4 DNA is protected from most restriction endonucleases due to the HMC resides (3). T4 also modifies the host RNA polymerase to preferentially bind HMC DNA, but this would not inhibit T7 replication as it codes its own RNA polymerase which it uses for the majority of its replication cycle (2) These potential modes of competition may play out equivalently during a co-infection and result in an overall titer drop, but without one phage clearly out-competing the other.

Theoretically, either phage could encounter any given host cell first and potentially exclude the other. If this were the case, it was expected that T4 would have been the dominant phage, demonstrated by a higher titer of progeny virus in the co-inoculation at 90 min. This expectation stems from the superinfection exclusion mechanism that T4 is able to employ within approximately 4 minutes after infection. T4 is able to cause DNA from other related phages to be released into the periplasm and be excluded from entrance into the host cell (T4 genome review). If this mechanism was also successful against T7, there would have been

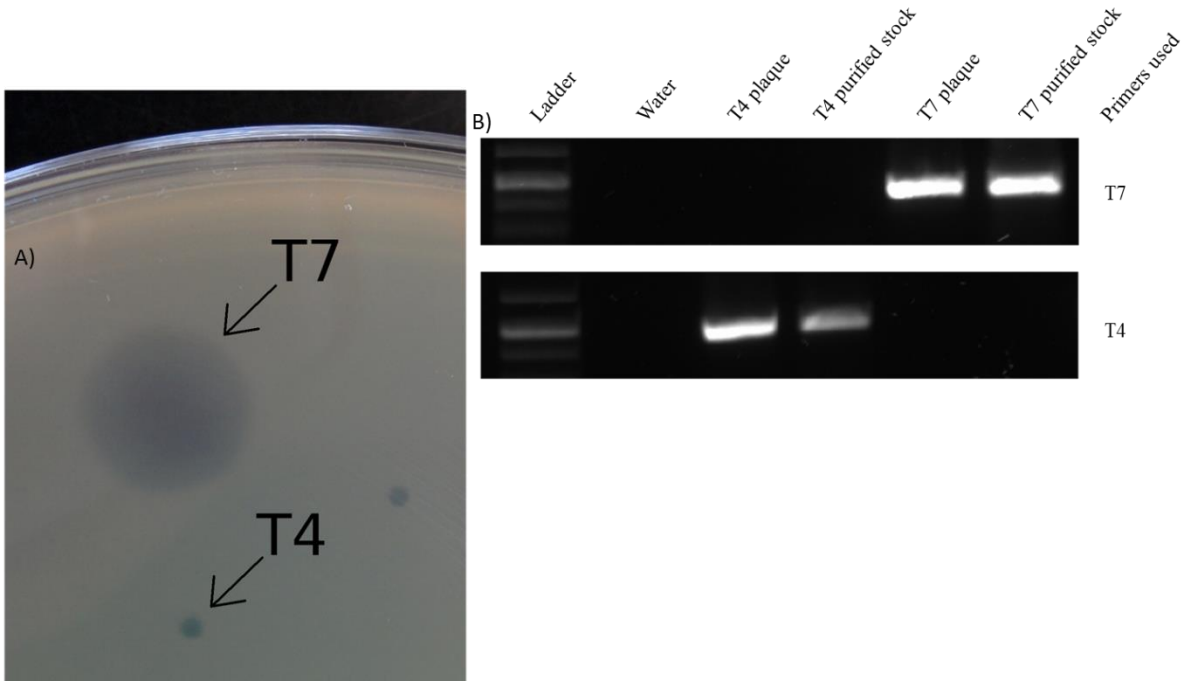
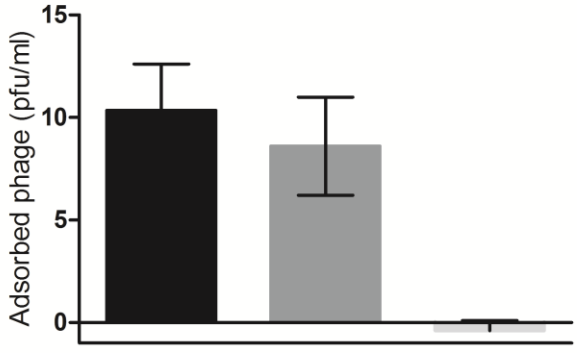


FIG 2 A) Representative plaque morphologies for T7 and T4 on *E. coli* B23. B) PCR results of representative plaques and purified stocks using specified primers. The bright band in each ladder corresponds to the 500 bp band in theThermo Scientific 1kb Gene Ruler plus molecular ladder.

significantly less T7 in the co-inoculation than T4. It is possible that this effect only works against other T4 and related T-even phages.

In conclusion it was observed that co-inoculation of T4 and T7 in *E. coli* B23 decreased the overall phage titer produced at 90 min. Interference between the two phages was clearly demonstrated however the exact mechanism by which this occured remains elusive.

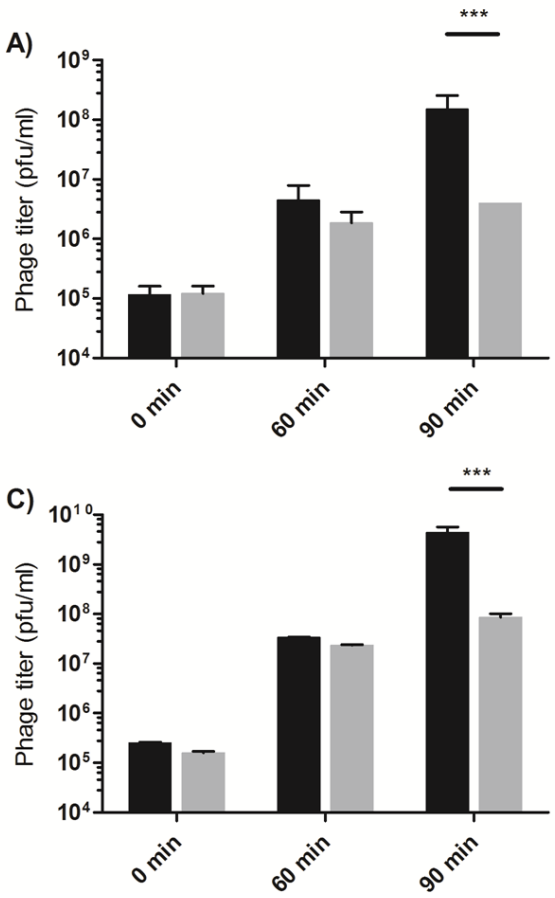


F IG 3 The effect of the strain of *E. coli* host cells on the adsorption of T4 over 12.5 minutes. *E. coli* B23 ( ), C600 ( ) and C ( ). Presented values were multiplied by $\times 10^{-5}$.

FUTURE DIRECTIONS

As observed in this experiment, there were two bursts. In order to ensure that the experiment carried out is indeed a one-step assay and eliminate conditions for a second burst, one future experimental modification is to verify that all *E. coli* cells are lysed and unable to replicate, thereby not creating more hosts for phage infection. This is to ensure competition for host cells occurs between the phages. This can be done by sampling the bacteria and plating for colony forming units at the same time points as the one step double overlay assay. This assay would allow the experimenter to track the rate of death along with the rate of titer increase within the virus at identical time points. This would indicate if the phages are lysing identical numbers of cells between conditions, or if lysis is impaired.

Another experimental modification that could be considered is to use fluorescent tagging. A plasmid containing both a Green Fluorescent Protein (GFP) under the control of a T7 promoter and a Red Fluorescent Protein (RFP) under the control of a T4 promoter could be inserted into the *E. coli*. GFP would only be expressed if a T7 infects the cell, since T7 encodes for its own RNA polymerase (2). Conversely, T4 uses host polymerase; nevertheless, since T4 infection causes a modification of the host polymerase to only transcribe T4 genes, then RFP expression would occur exclusively during a T4 infection (1). Fluorescence Assisted Cell Sorting (FACS) could be used to determine if there are cells expressing both GFP and RFP, thereby verifying that there was true co-infection. Post FACS the separated mono-infected and co-infected cells could be introduced into their own *E. coli* B23 cultures in logarithmic growth and allowed to replicate. A normal one step double overlay plaque assay would be performed through a time period of 60 minutes. This experiment would definitively show whether co-infection of the same cell by two phages occurs and the effects it has on burst size and timing.



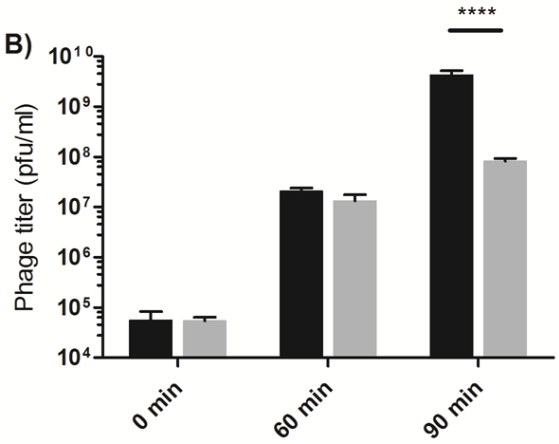


FIG 4 Comparison of viral titer between mono-infection (■) and co-inoculation (▒) using T4 titer only (A), T7 titer only (B), or the combined titer of T4 and T7 (C) in *E. coli* B23. Statistical analysis done by two-way ANOVA, *** denotes $p<0.005$, **** denotes $p<0.0001$.

To further our understanding of the mechanism of competition between T4 and T7, an experiment that could be carried out is a delayed co-inoculation one-step assay in which T4 is added to infect the host cells before adding T7, and then the equivalent one-step assay where T7 is added before T4. In this way, with one phage being able to adsorb before the other, one would be able to determine whether the observed competition is due to a competition in binding to host cells or to intracellular competition mechanisms. For example, if T7 were allowed to adsorb to host cells first and yet we still see T4 being able to produce progeny, then we could hypothesize that T4's intracellular competition mechanisms are fast-acting and that T7 binding does not exclude T4 binding. On the other hand, if T7 binds first and we see no or a decrease in T4 plaque forming units, then we can conclude that T7 binding is either sufficient to exclude T4 from binding, or that T4 *is* able to co-infect but its intracellular competition mechanism is not fast enough to outcompete a T7 phage given a head start; this latter scenario would suggest that that either entry is the more important mechanisms of competition or a combination of entry and intracellular mechanisms are required for competition. In fact, Loewen observed that T7 excluded T4 when T4 super-infected *E. coli* 5 minutes or more after T7 infection (13).

To further our understanding of the mechanism of competition between T4 and T7, an experiment that could be carried out is a delayed co-inoculation one-step assay in which T4 is added to infect the host cells before adding T7, and then the equivalent one-step assay where T7 is added before T4. In this way, with one phage being able to adsorb before the other, one would be able to determine whether the observed competition is due to a competition in binding to host cells or to intracellular competition mechanisms. For example, if T7 were allowed to adsorb to host cells first and yet we still see T4 being able to produce progeny, then we could hypothesize that T4's intracellular competition mechanisms are fast-acting and that T7 binding does not exclude T4 binding. On the other hand, if T7 binds first and we see no or a decrease in T4 plaque forming units, then we can conclude that T7 binding is either sufficient to exclude T4 from binding, or that T4 *is* able to co-infect but its intracellular competition mechanism is not fast enough to outcompete a T7 phage given a head start; this latter scenario would suggest that that either entry is the more important mechanisms of competition or a combination of entry and intracellular mechanisms are required for competition. In fact, Loewen observed that T7 excluded T4 when T4 super-infected *E. coli* 5 minutes or more after T7 infection (13).

TABLE 2 Reduction in titer as a result of co-inoculation.

Type of phage titre following co-inoculation with both phages	Comparative reduction in titer (%) at different times[a]	
	0 min	90 min
T4	41	97
T7	33	98
Summed (T4 and T7)[b]	37	98

[a] Values expressed are the percent reduction of mono-infection titer at each time point.

[b] Summed compares the summed titer from co-inoculation to the summed titer of both individual T4 and T7 mono-infections.

ACKNOWLEDGEMENTS

We would like to thank Dr. William Ramey, Dr. David Oliver, and Richard White for their guidance. We would also like to

thank the Department of Microbiology and Immunology of the University of British Columbia for financial support. Finally, we extend our gratitude to the staff of the UBC Wesbrook Building media room.

REFERENCES

1. **Mosig G, Eiserling F**. 2006. T4 and Related Phages: Structure and Development, p. 226. *In* The Bacteriophages, 2nd ed. Oxford University Press, London.
2. **Molineux IJ**. 2006. The T7 Group, p. 227. *In* The Bacteriophages, 2nd ed. Oxford University Press, London.
3. **Miller ES, Kutter E, Mosig G, Arisaka F, Kunisawa T, Rüger W**. 2003. Bacteriophage T4 genome. Microbiol. Mol. Biol. Rev. **67**:86-156.
4. **Alter MJ.** 2006. Epidemiology of viral hepatitis and HIV co-infection. J. Hepatol. **44**:S6-S9.
5. **Barker H**. 1989. Specificity of the effect of sap-transmissible viruses in increasing the accumulation of luteoviruses in co-infected plants. Ann. Appl. Biol. **115**:71-78.
6. **Ling CM, Hung PP, Overby LR**. 1970. Independent assembly of Qβ and MS2 phages in doubly infected *Escherichia coli*. Virology. **40**:920-929.
7. **Chan S, Shi R, Tang T, Wang M**. 2013. T4 bacteriophage dominates T7 bacteriophage during co-infection of *Escherichia coli* C600. J. Exp. Microbiol. Immunol. **17**:125-128.
8. **Kropinski AM, A Mazzocco, TE Waddell, E Lingohr, RP Johnson.** 2009. Enumeration of bacteriophages by double agar overlay plaque assay, p. 69. *In* Bacteriophages methods and protocols volume 1: isolation, characterization, and interactions. Humana Press, New York.
9. **Adams MH**. 1959. Effects of physical and chemical agents (except radiation) on bacteriophage particles, p. 53. *In* Bacteriophages. Interscience Publishers, Inc., London.
10. **Stent GS.** 1963. Growth cycle, p. 72. *In* Molecular biology of bacterial viruses. W. H. Freeman and Company, San Francisco.
11. **Carolina Biological Supply Company**. 2004. Techniques for studying bacteriophages of *Escherichia coli*: Reference manual. Carolina Biological Supply Company, Burlington, NC.
12. **Delbruck M**. 1945. Interference between bacterial viruses: III. The mutual exclusion effect and the depressor effect. J. Bacteriol. **50**:151-170.
13. **Loewen RR**. 1978. Mixed bacteriophage infection of *Escherichia coli*: exclusion of superinfecting T4 by T7. M.Sc. thesis. University of British Columbia, Vancouver, BC.