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Evaluating Double Agar Overlay Assay and Flow Cytometry as Methods for Characterizing Competition between T4 and T7 Bacteriophages in *Escherichia coli* C600

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Bacteriophages are the most abundant viruses found on earth with population densities approximately ten fold of prokaryotes in the same environments. As a result, interspecies competition likely plays a large role in regulating phage populations in the environment. T4 and T7, two of the most well characterized bacteriophages, infect *Escherichia coli*. Co-infection experiments using *E. coli* can be used to study the interspecies competition between these phages. Previous work studying competition dynamics through respective phage population fluctuations reported contradictory data in T7 and T4 co-infection experiments. However, variables such as time of incubation of cells with phage and multiplicities of infection varied thereby confounding comparisons. We sought to establish a reliable experimental system to address these discrepancies in order to study phage population dynamics during co-infection of *E. coli*. Mono-infection and co-infection experiments using T4 and T7 phage were performed. Phage progeny were enumerated by observing phage plaques in double agar overlay assays. T4 and T7 plaque sizes were expected to be different thereby enabling enumeration of each phage. We observed variable plaque morphology within pure phage populations of T7 of mono-infected culture. These data suggest that double agar overlay may not be a reliable method to enumerate T7 in co-infection experiments. We therefore pursued a flow cytometry based enumeration approach as an alternative method to characterize phage competition. Our data suggest that flow cytometry can be used to measure changes in phage populations. Preliminary results of co-infection experiments using flow cytometry suggest that T4 phage outcompete T7 phage during co-infection of *E. coli* C600 at a multiplicity of infection of 0.25 for each virus.

Metagenomic analyses estimate the number of phage particles in the biosphere to be 10^{30}, which is an order of magnitude greater than the prokaryotic population of the planet (1,2). Despite the abundant viral sequence data gathered from environmental samples, current databases offer little information on phages not of T4 or T7 type families (2). As such, a knowledge deficit exists within the scientific research community in characterizing environmental phages and the resulting implications in allocation of resources within ecosystems. Paradigm shifts such as the incorporation of the microbial loop, wherein phages prevent dissolved organic carbon to reach high trophic levels in marine microbial food webs (3) exemplify the revision required of ecological concepts as our knowledge of environmental phages continue to increase. While research on the impact of phages on other members of the environment is still in infancy, knowledge of phage competition for shared resources and hosts is comparatively null. Selection pressure has been established as a causative agent for driving co-evolution of competing populations, facilitating horizontal gene transfer and transformation of entire ecological community compositions (4). Thus, when combined with the sheer magnitude of environmental phages, research on phage competition may have far reaching implications in microbial ecology similar to the development of the aforementioned microbial loop model. Previous work on phage competition has been pursued exclusively in the context of accessible and well characterized laboratory phages T4 and T7 through the shared host *Escherichia coli.*

T4 and T7 Enterobacteriophages belong in the collective order of Caudovirales, assigned to dsDNA nonenveloped viruses (2). T4 bacteriophage, of family Myoviridae, is a relatively large lytic phage with a genome of 169 kbp (5). Infection cycles typically last 25 minutes, and the burst size is approximately 125 particles per infected host (5). When plated, plaques have been described as small pointed pinpricks (6). T7 bacteriophages, of the Podoviridae family, are considerably smaller and harbor a 40 kbp genome (7). Infection cycles can vary in relation to environmental conditions but lysis can be observed at roughly 17 minutes, yielding approximately 100 phages per infected cell (8). Plaques are observed to be large circles (0.5 – 2 cm) with diffused halos (6).

Using a three component system of *E. coli* as resource and T4 and T7 phages as competitors, previous research provided conflicting results in competition outcome and winner determined by the phage with higher particles at a given time. Chan *et al.* (9) inoculated *E. coli* C600 with variable phage to cell ratios (multiplicity of infection, MOI) of T4 and T7 and allowed 5 minutes of incubation prior to harvesting media supernatant for respective phage enumeration. Two conditions were observed: one in which T4 MOI was 1.2 and T7 MOI was 0.5 and the other condition with MOIs reversed. In all situations of variable MOIs in co-inoculation, T4 phage has been observed to robustly outcompete T7 phages in phage progeny produced under competitive stress during the initial stage of virus adsorption into the host cell. Using *E. coli* B600 as a host strain, Nguyen *et al.* (6) concluded that neither phage outcompeted the other. However, this study used an MOI of

5 for each phage and measured phage progeny at 0, 60, and 90-minute time points post infection (6), which may in part explain how the two studies reached different conclusions. Respective to mono-infected controls, Nguyen *et al.* observed an overall ~95% decrease in phage titer for both phages and thus concluded neither phage succeeded in outcompeting the other.

In order to be able to conduct quantitative assessments of phage competition, a detailed understanding of the assay method is required. The double agar overlay assay (DAOA) method has historically been used to enumerate phage populations by counting phage plaques formed on a lawn of bacteria. A useful phage enumeration assay should be accurate, precise, sensitive, and efficient.

Here, we have assessed the DAOA as a method to enumerate phage. We concluded that the assay does not accurately distinguish T7 from T4 and it is not efficient. We present flow cytometry as an alternative approach to enumerate phage. Using flow cytometry, we present initial data from a co-infection experiment conducted with T4 and T7 bacteriophages.

MATERIALS AND METHODS

Strains used in this study. *E. coli* B23, *E. coli* C600, and bacteriophages T4 (Carolina Biological Supply 124330), ϕX174 (Carolina Biological Supply 124425), and T7 strains were all obtained from the Microbiology 447 culture collection from the department of Microbiology and Immunology, University of British Columbia.

Generation of primary phage lysates. Adapted from Mosig, G (5). In order to propagate substantial quantities of T4 and T7 bacteriophages, primary cell lysates were obtained from infections of 5 ml of log phase *E. coli* B23 grown to an optical density at 600 nm (OD_{600}) of 0.125 with 5 µl of the aforementioned commercial phage stocks. OD_{600} measurements were made using the Pharmacia Biotech Ultrospec 3000. The culture was incubated in a 37℃ shaker for 8-10 hours to achieve complete lysis. Following incubation, 150 µl of chloroform was added to encourage lysis of any remaining bacterial cells. The lysate was subsequently left overnight at room temperature, and the aqueous fraction was removed the following morning. 150 µl of chloroform was added to the lysate and stored at 4℃ until use. Usage of *E. coli* B23 strain permitted exclusion of ΦX174 phage strain contamination.

PCR amplification of phage capsid genes to determine purity of phage extraction. PCR amplification of T4 and T7 genes was performed using Platinum® Pfx DNA Polymerase (Invitrogen) kit components as per the manufacturer's instructions. Primers were ordered from Integrated DNA Technologies (IDT) and were used at final concentrations of 0.1 µM per reaction sample. The Whatman Biometra T-Gradient Thermocycler was programmed for a 10-minute initial denaturation step at 95℃, followed by 30 cycles of 5 minutes of denaturation at 95℃. In the annealing phase of 30 seconds, the T7 primers required a temperature of 53℃ and T4 primers required 51℃ as experimentally determined by gradient PCR. Extension phase for both primer reactions occurred at 74℃ for 30 seconds. PCR products were run on a 1.2% agarose gel in 1X TAE buffer at 110 V for 30 minutes. Bands were visualized using ethidium bromide.

Double agar overlay assay for phage enumeration. Reagents were prepared as outlined in the standard protocol by Kropinski *et al.* (10). *E. coli* B23 was shown to better resolve plaque morphology for T4 and T7 (6). As such, this strain was used as the indicator bacterium for this assay. The bacteria were grown to an OD_{600} of 0.125, poured into heated 7% Luria agar (1 mM $CaCl_2$) overlay, and mixed with variable dilutions of phage infected bacterial cultures or with the phage stock. The mixtures were then poured over 15% Luria agar (1 mM $CaCl_2$), allowed to solidify and incubated overnight at 37℃. Plaques were counted under the assumption that one plaque forming unit (pfu) represents one phage isolated from the infected *E. coli* supernatant.

Mono-inoculation of T4 and T7 bacteriophages with *E. coli* C600. *E. coli* C600 were grown to 0.125 OD_{600}, inoculated with titrated T4 or T7 phage stocks at an MOI of 0.5 and incubated in 37℃ shakers. In ten minute increments, 1 ml culture samples were taken for OD_{600} measurement and phage enumeration. Phage were isolated through centrifugation of culture samples at 4000 x g for 15 minutes. The resulting supernatant was used in the DAOA for phage enumeration as outlined above. For replicate mono-inoculations, additional time samples were taken 5 minutes before and 5 minutes after the peak OD_{600} was observed in order to identify when lysis of the bacteria commenced.

Evaluation of plaque morphology and identity with *E. coli* B23. Plaques obtained from DAOA of mono-inoculations were used as templates for PCR amplification in order to verify phage identity. The plaques were stabbed with a sterile needle or pipette tip. The tip was used to transfer the template DNA into the PCR reaction tube. A 1:1 ratio of T4 and T7 phages were mixed and plated with *E. coli* B23 to observe the discernibility of respective phage morphologies and accuracy of the assay. All relevant plaques were imaged using the Alphaimager Multiimage Light Cabinet.

Flow cytometric detection and enumeration of T4 and T7 phages. Preparation of samples is based on the protocol outlined by Brussaard (11). Harvested phage-containing samples were passaged through 0.2 µm filter (Millipore) and treated with 0.5% v/v of glutaraldehyde (Sigma) for fixation. Samples were frozen immediately with liquid nitrogen and stored for analysis in -80°C. After thawing and diluting 10^3-10^4 fold in 0.2 µm filter sterilized TE buffer (pH 8), samples were stained with SYBR Green and incubated at 80°C for 10 minutes prior to analysis with the FACscalibur flow cytometer (Becton-Dickinson). Phage were enumerated as a function of cytometer flow rate of sample volume, sample dilution and number of positive events within gated region of interest.

Co-inoculation of T4 and T7 phages with *E. coli* C600. Co-inoculations of T4 and T7 phages were performed following identical protocols to mono-inoculations, subsequent to the addition of a summative phage MOI of 0.5 (MOI 0.25 for each phage), in *E. coli* C600 grown to an approximate OD_{600} of 0.16. In anticipation of sudden depletion of cells, co-inoculated cultures were given 1 ml aliquots of stationary phase *E. coli* (OD_{600} 0.160) at ten-minute time intervals. This extraction was done simultaneously with the removal of two 1 ml samples for flow cytometric analysis and optical density readings every ten minutes in order to limit the amount of time the cultures were exposed to the external environment. Mono-inoculations of each phage were run in parallel without the addition of extra cells to provide single phage controls for flow cytometry that were generated and fixed in the same time frame. Complete lysis was defined by identical optical density readings gathered from one time point to the next, indicating the end of the infection cycle.

RESULTS

Propagated phage from stock were titered using DAOA, with purity determined via PCR and gel electrophoresis. To confirm concentration of bacteriophage present in commercial stocks, primary lysates were generated and enumerated using the DAOA. Two T4 commercial stocks

were obtained for T4 primary lysate propagation, while a single T7 commercial stock was used to generate a T7 primary lysate. PCR and gel-electrophoresis was performed to confirm the identity and purity of the phage lysates. Previous work in our lab was hindered by contamination of T4 and T7 commercial phage stocks with the ϕX174 phage (Carolina Biological Supply 124425). PCR conditions were optimized for ϕX174 primers using commercial ϕX174 phage stocks as positive control. These primers were then used to assess contamination of T4 and T7 primary lysates and resulting downstream experiments. Gel analysis confirmed the identity and purity of the T7 lysate (Fig. 1a). Interestingly, only one of the two T4 lysates were identified as pure T4 bacteriophage (Fig. 1b). Identity of the second lysate could not be confirmed via PCR analysis utilizing the primers identified in Table 1 (Fig. 1a, b). This unidentified phage lysate (P^u) was stored for further analysis. Using the DAOA, plates with countable numbers of plaques were counted in duplicate and averaged, yielding values of 7.65 x 10^{10} pfu/ml for the T7 lysate and 7.3 x 10^9 pfu/ml for the T4 lysate.

TABLE 1 Primers designed for amplifying fragments of φx174 major coat gpF, T4 major capsid gp23, T7 capsid gp10a. The letters F and R denote the forward and reverse primers, respectively.

Gene	Sequence (5' – 3')	Size (bp)
φx174 gpF	F: ACGGACTGGAAACACTGGTC	476
	R: CGCTCTAATCTCTGGGCATC	
T4 gp23	F: GCCATTACTGGAAGGTGAAGG	398
	R: TTGGGTGGAATGCTTCTTTAG	
T7 gp10a	F: CGAGGGCTTAGGTACTGC	295
	R: GGTGAGGTGCGGAACTTC	

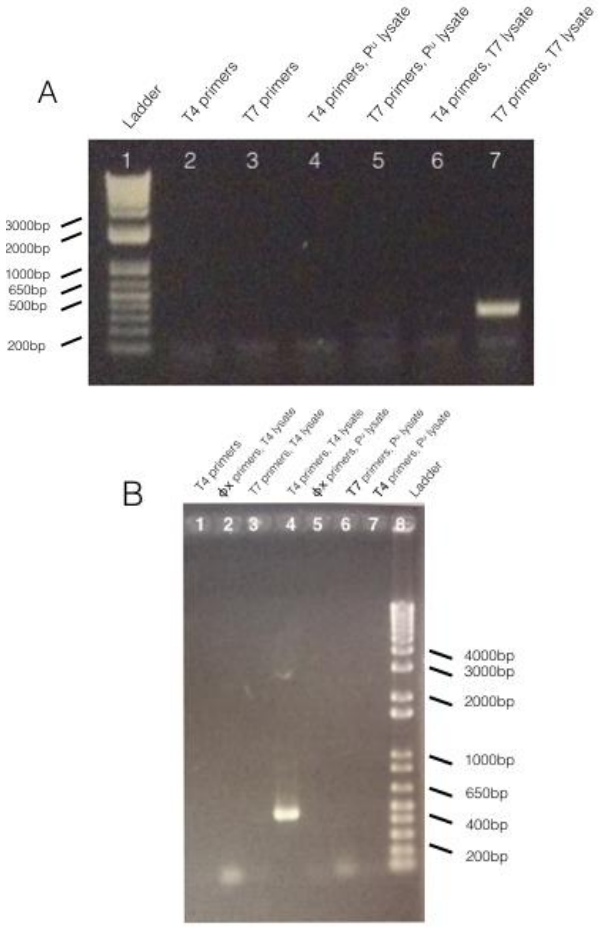

FIG. 1. T4 and T7 primary lysates are not contaminated with laboratory phage strains. (A) 1 kb DNA ladder in lane 8 was used to determine the relative size of the bands. Lanes 2 and 3 represent a negative control for T4 and T7 respectively. Lanes 4 and 5 contain the P^u primary lysate tested with T4 and T7 primers respectively. Lanes 6 and 7 represent T7 primary lysate tested with T4 and T7 primers respectively. (B) 1 kb DNA ladder in lane 8 was used to determine the relative size of the bands. Lane 1 represents a negative control for the T4 primer set. Lanes 2, 3 and 4 represent the T4 primary lysate tested with ϕX147, T7 and T4 primer sets, respectively. Lanes 5, 6 and 7 contain the P^u primary lysate tested with ϕX147, T7 and T4 primer sets, respectively.

The double agar overlay assay yields functionally relevant data for phage life cycles in mono-inoculations. DAOA was used as an assay to study phage life cycles. Mono-inoculation experiments were performed in duplicate for both T4 and T7. Only one replicate for each is displayed, as the procedure was being optimized throughout the project. The graphs shown below (Fig. 2a, b), were selected as they generated results that agreed with previous findings in the literature. Cell mortality and phage growth in mono-inoculations were monitored through OD_{600} readings and sample isolations for DAOA. T7 mediated cell lysis appeared uniformly over the course of the inoculation, as shown by the gradual rise in viral titer (Fig. 2a). Phage titer plateaued concurrently with complete lysis of the bacteria. For the T4 phage life cycle (Fig. 2b), a distinct drop in phage titer during the first 20 minutes was observed, indicating adsorption of the phage particles to the bacteria. The phage titer subsequently increased until 40 minutes, at which time the viral titer plateaued. A second dip in titer is observed at 80 minutes, which is likely due to experimental error. Notably, OD_{600} measurements for T4 mono-inoculations declined at a slower rate than in T7 experiments. In both cases cell lysis began at approximately 20 minutes, yet complete lysis for T4 was observed at 120 minutes, compared to 40 minutes for T7. During the T4 mono-inoculation, an uninfected bacterial culture was incubated in parallel as a control (data not shown). The OD_{600} for this culture increased throughout the entirety of the experiment, as expected. These results indicate phage-mediated cell lysis and phage growth can be successfully monitored in mono-inoculations using DAOA.

The double agar overlay does not differentiate phage particles in co-inoculations due to variable plaque morphology of T7 in *E. coli* B23. Samples from T4 and T7 mono inoculations were plated via the DAOA and differential plaque morphology was assessed. T4 plaques formed small, punctate, circular plaques ranging 0.5-1 mm in diameter (Fig. 3b). Unexpectedly, the plaque morphology of P^u, the purported T4 lysate, was contrasting to that of T4 and appeared similar in morphology to T7 (Fig. 3c). T7 formed circular plaques with central clearings surrounded by translucent halos. The diameter of T7 plaques was variable, ranging from 2-7 mm (Fig. 3a). Fluctuations in T7

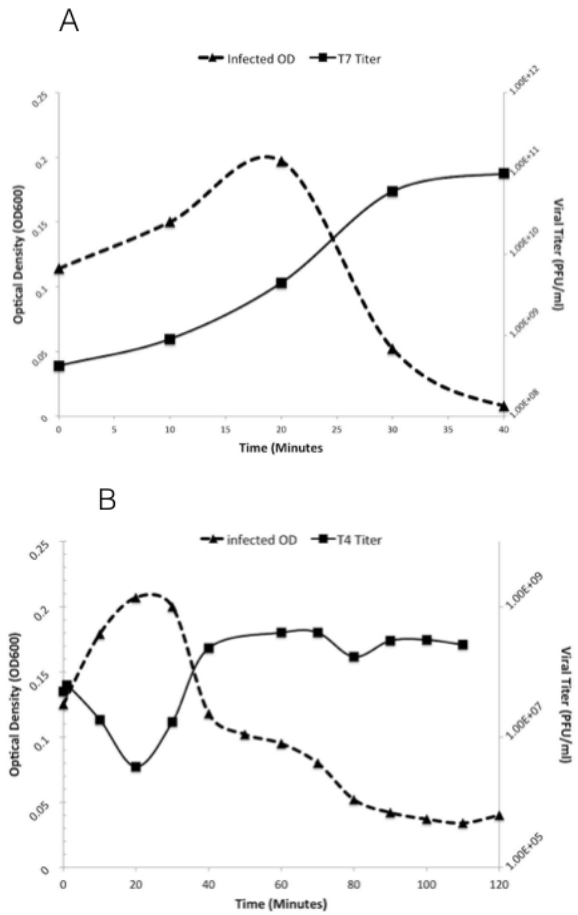

FIG. 2. The DAOA and OD_{600} adequately show bacteriophage life cycle dynamics in a mono-inoculated *E. coli* C600 culture at a MOI of 0.5 (starting OD_{600} = 0.125). (A) Bacteriophage T7 mono-inoculated culture showing increasing viral titer and bacterial cell lysis during a time course infection. (B) Bacteriophage T4 mono-inoculated culture showing primary absorption at 20 minutes via the drop in OD_{600}. Mono-inoculated culture also displays increasing viral titer and bacterial cell lysis during a time course infection.

plaque morphology suggested cross contamination between phage stocks. Samples were taken from T7 plaques with differential diameters and PCR was performed to confirm plaque identity. Despite varying morphology, PCR and gel-electrophoresis showed that the plaques were representative of T7 phage, and cross contamination with T4 did not occur (Fig. 3e). These results were unexpected, as plaque morphology is often utilized to identify T4 and T7 phage in co-inoculation experiments. To test whether plaque morphology could be used to identify phage in a co-inoculated environment, a 1:1 ratio of T7 and T4 phage were mixed and plated using the DAOA. Larger T7 plaques were easily visualized amongst the small punctate T4 plaques, however, plaques that may have represented small T7 or large T4 plaques could not be distinguished (Fig. 3d). In addition, despite the equal ratio of T7 to T4 phage particles, T4 plaques heavily outnumbered T7 plaques. These results suggest that the DAOA does not accurately visualize the ratios of mixed phage populations used to infect indicator bacteria and thus, usage of the method will be rife in imprecision and insensitivity in a co-inoculation setting.

Flow cytometry can differentiate between T4 and T7 phage populations. Discrepancies observed in phage morphology and quantification using the DAOA lead us to investigate flow cytometry as an alternative method for the analysis of co-inoculations. Populations were visualized using the DNA stain SYBR Green, and were distinguished based on differential genome sizes. T4 virus like particles (VLP) were identified as green fluorescence^{hi} and T7 VLP as green fluorescence^{low}. A negative control of TE buffer yielded background in two areas in the absence of phage (Fig. 4a). The population with a high degree of green fluorescence and side-scatter is likely due to bacteria, which is unexpected since this population is absent from the rest of the samples (Fig. 4b-e). The secondary background population was identified as green fluorescence^{low} and is likely due to insufficient filtration of TE and Glutaraldehyde. This background population (green fluorescence^{low}) was also present in the T4 mono-inoculation positive control (Fig. 4b). This population is easily distinguishable from fluorescence^{hi}, the expected T4 population. However, considering this background population (green fluorescence^{low}) lies where we expect to see the T7 population, this may also suggest T7 contamination in the T4 lysate. To assess whether this was the case, the T4 lysate was plated and plaque morphology was observed (data not shown). All plaques were consistent with expected T4 morphologies, suggesting that there was no cross-contamination. However, due to the varying plaque morphology previously observed using this assay, we are not fully confident with this result. In the T7 positive control (Fig. 4c), one population was observed at green fluorescence^{low}, as expected. However, considering background overlaps with green fluorescence^{low}, it is impossible to distinguish between background fluorescence and the T7 population using this readout. To investigate this, T7 gates were constructed on both the negative and T7 positive controls. The frequency of events found within this gate was higher in the T7 positive control relative to the negative control (Fig. 4f). This suggests that a T7 population is present within this gate. To compensate for background fluorescence, the frequency of events within the T7 gate of the negative control was subtracted from the events resolved in the T7 gate of the positive control and co-inoculated samples (Fig. 4e). This compensated total was used to calculate VLP/ml. Gates were kept consistent for all samples. These results show that flow cytometry can be used to differentiate between T4 and T7. However, sample preparation needs to be optimized in order to minimize background fluorescence, which will allow for the assay to be more sensitive.

Preliminary flow cytometry results suggest that T4 (MOI 0.25) outcompetes T7 (MOI 0.25) in a co-inoculation of *E. coli* C600 at a summative MOI of 0.5. To facilitate interpretation of T4 and T7 co-inoculations, time course readings of OD_{600} and VLP/ml were examined. OD_{600} readings of co-inoculated cultures were obtained in duplicate and visualized in Figure 5a. This figure shows

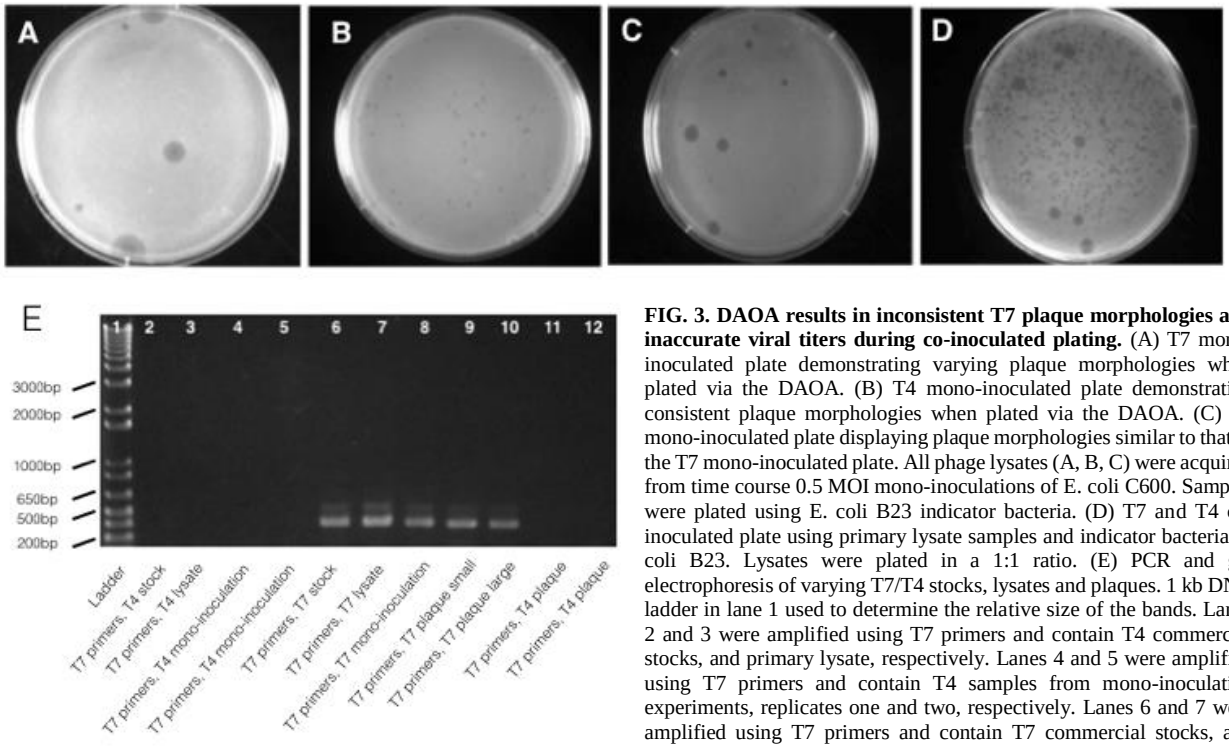

FIG. 3. DAOA results in inconsistent T7 plaque morphologies and inaccurate viral titers during co-inoculated plating. (A) T7 mono-inoculated plate demonstrating varying plaque morphologies when plated via the DAOA. (B) T4 mono-inoculated plate demonstrating consistent plaque morphologies when plated via the DAOA. (C) Pu mono-inoculated plate displaying plaque morphologies similar to that of the T7 mono-inoculated plate. All phage lysates (A, B, C) were acquired from time course 0.5 MOI mono-inoculations of E. coli C600. Samples were plated using E. coli B23 indicator bacteria. (D) T7 and T4 co-inoculated plate using primary lysate samples and indicator bacteria E. coli B23. Lysates were plated in a 1:1 ratio. (E) PCR and gel electrophoresis of varying T7/T4 stocks, lysates and plaques. 1 kb DNA ladder in lane 1 used to determine the relative size of the bands. Lanes 2 and 3 were amplified using T7 primers and contain T4 commercial stocks, and primary lysate, respectively. Lanes 4 and 5 were amplified using T7 primers and contain T4 samples from mono-inoculation experiments, replicates one and two, respectively. Lanes 6 and 7 were amplified using T7 primers and contain T7 commercial stocks, and primary lysate, respectively. Lane 8 was amplified using T7 primers and contain T7 lysate from a mono-inoculation experiment. Lanes 9 and 10 were amplified using T7 primers and contain samples taken from small and large T7 plaques respectively. Lanes 11 and 12 were amplified using T7 primers and contain samples taken from T4 plaques.

OD_{600} rising for 30 minutes, followed by a sharp decline for the next 30 minutes. From this point onward, OD_{600} measurements drop incrementally until the complete lysis is observed at approximately 120 minutes. This trend is consistent between both trials, and it also closely mirrors the OD_{600} curve observed in the T4 mono-inoculated culture (SFig. 2). Supplemental *E. coli* 600 were added to the culture in ten-minute intervals during co-inoculation experiments. This was to ensure host cells were not limiting during co-inoculation and allowed for full visualization of the competitive interaction between phage. VLP/ml concentrations were calculated using data acquired from flow cytometry experiments. VLP/ml was calculated using the experimental flow rate (ul/min) and total events within desired population gates. The results of these calculations are represented in Figure 5b. This data demonstrates T4 maintaining a lead in phage population during the first 40 minutes of the co-inoculation. A drop in phage VLP/ml is observed at t=60, which may suggest reabsorption of phages or possible experimental error. The later time points reveal T4 increasing in VLP/ml and peaking at 1.2×10^{11} VLP/ml at 100 minutes. On the other hand, the T7 counts are substantially lower, peaking at 4×10^{10} VLP/ml. A dip in VLP/ml is observed for both T4 and T7 at 120 minutes, which could be due to experimental error. Only one co-inoculation experiment was performed due to time constraints. As such, further replicates need to be performed before making conclusions about the outcome of competition. That being said, our data suggests that T4 ultimately outcompetes T7 in a co-inoculation at a summative MOI of 0.5 over the course of 120 minutes.

DISCUSSION

The goal of this study has been to characterize competition dynamics between T4 and T7 bacteriophages in a co-inoculated environment using *E. coli* C600 as shared resource. The DAOA was initially used as a means to enumerate phage populations. While this assay was useful for mono-inoculations, the variability in plaque morphology as well as the enumeration of plaques, suggested that co-inoculation studies using the DAOA method would not yield reliable data. Flow cytometric detection was therefore used as an alternative method to enumerate T4 and T7 phage in cell lysates. Flow cytometry was found to be more suitable method for this purpose due to its efficiency and ability to consistently differentiate between the phage species. Using this method, preliminary data suggests that T4 outcompetes T7 in a co-inoculation of *E. coli* C600 at a summative MOI of 0.5.

The utility of the DAOA in its ability to characterize competition was assessed through performing both mono and co-inoculations. The data generated from the mono-inoculations (Fig. 2a, b) display the progression of the lytic cycle for each phage; indicating points of adsorption, bursts of phage from the bacteria, and delayed lysis in the T4 infected culture. Mono-inoculations These observations indicate that the

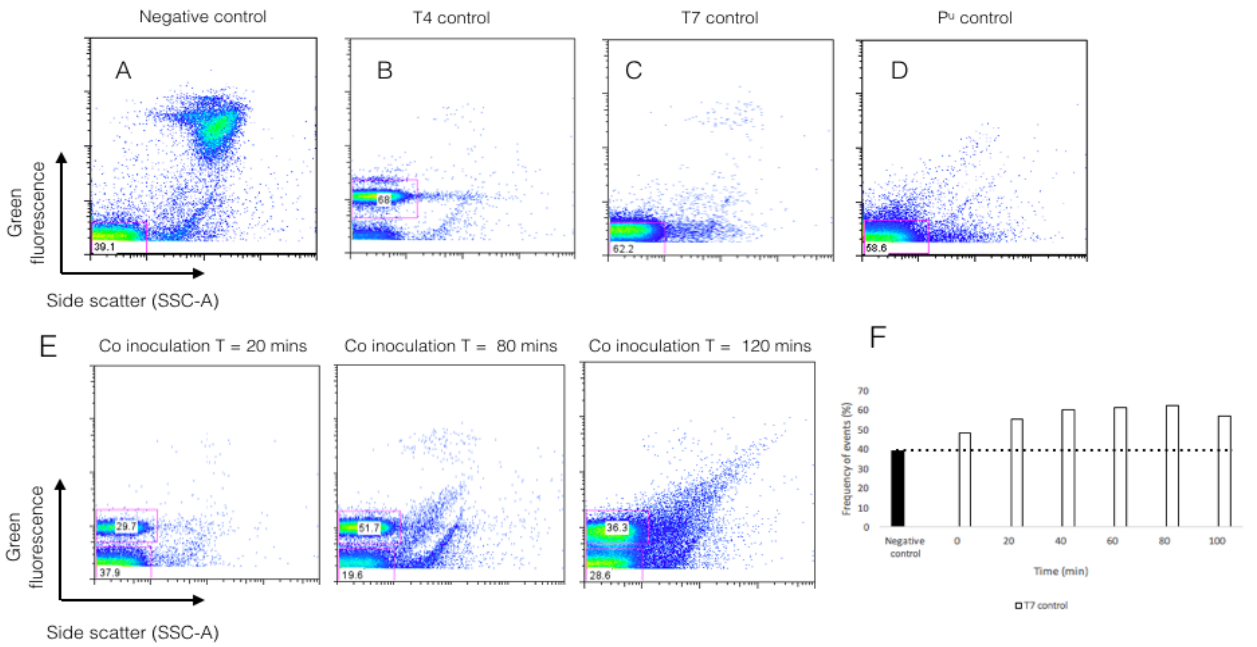

FIG. 4. T4 bacteriophage populations are easily identified via flow cytometry while T7 populations are require compensation due to background fluorescence. (A) Negative control flow plot demonstrating background fluorescence (Green fluorescencelow) and potential bacterial contamination (Green fluorescencehi, SSC-Ahi). (B) Positive control plot for T7 bacteriophage. (C)Positive control plot for T4 bacteriophage. Positive controls were acquired via mono-inoculations of E. coli C600. (D) Representative flow plot of Pu bacteriophage primary cell lysate. (E) Representative flow plots of T4/T7 co-inoculation experiments at time points 20, 80 and 120 minutes. Gating strategy for T4 (Green fluorescencehi) and T7 (Green fluorescencelow) and demonstrated. T7 frequency was compensated for potential background fluorescence using data acquired from the negative control plot. (F) Frequency of events observed in the T7 gate of time course T7 positive control samples in comparison to negative control.

functional relevance of the DAOA in displaying lytic phage cycle dynamics is sufficient when characterizing mono-inoculations. However, our experiments yielded inconsistencies in T7 plaque morphology (Fig. 3a). These disparities suggest that the DAOA may not be sensitive enough to correctly identify phage species through differential plaque morphology. Additionally, upon mixing equal amounts of T4 and T7 bacteriophages, the DAOA yielded substantially higher plaque counts for T4 compared to T7 (Fig. 3d). This may be due to the inability of the DAOA to differentiate between phage species based on plaque morphology. Additionally, another limitation may lie in the procedure of plating T4 and T7 phages simultaneously in an indicator bacterium. It could be suggested this method inherently gives rise to a second competitive interaction. To form a plaque, phage are required to adsorb to, replicate and lyse an indicator bacterial cell. Adsorption is the attachment of the phage to the host cell receptor which results in entry of the phage into the host cell. Chan *et al.* (9) previously observed that T4 phages outcompete T7 at the level of adsorption. It could be suggested that T4 sequester indicator bacteria after adsorption, making cells less available for T7 to be titrated. The DAOA may therefore be less accurate when plating more than one species of phage. These observations suggest that although the DAOA yields functionally relevant data in characterizing phage life-cycles, it is unable to differentiate between phage species, and it is not an accurate representative of phage titers. Thus, an alternative approach to characterizing competition dynamics in co-inoculations is needed.

Having ruled out the DAOA as suitable for analyzing co-inoculations, flow cytometry was investigated as an alternative approach as it is currently one of the standard tools in enumerating phage populations (11). In this method, populations are distinguished based on genome size and particle complexity (SSC) as opposed to plaque morphology. The near fourfold difference in genome size between T4 and T7 makes flow cytometry a viable strategy to differentiate between populations in co-inoculations (Fig. 4e). Additionally, the process of enumeration does not require an additional infection event as the DAOA does, and therefore reduces the likelihood of experimental error and confounding variable of added competition. Flow cytometry data was visualized by plotting green fluorescence, representing genome size, against SSC, representing particle granularity. Expected phage particles were suspected to have low granularity. Gates were constructed around prevalent populations visualized on these plots. The positive control for T4 (Fig. 4c) suggests T4 is

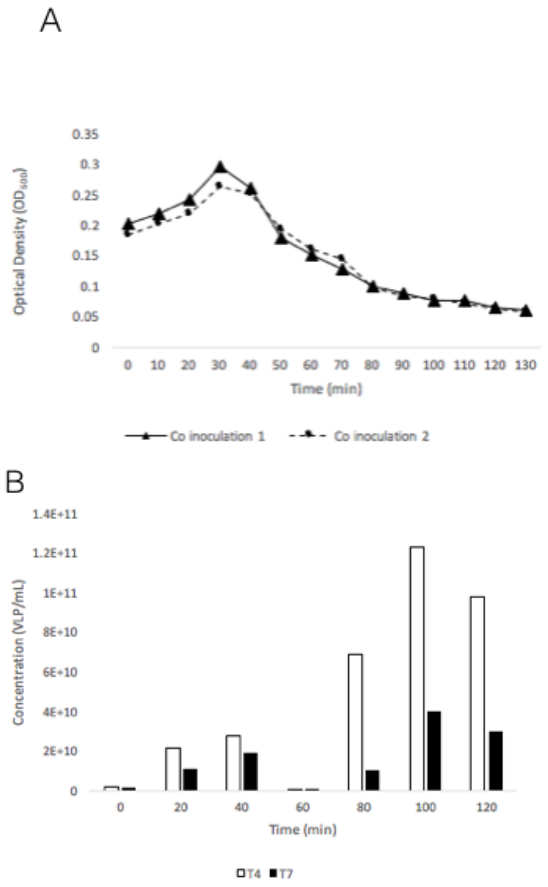

FIG. 5. Phage concentration and Optical Density measurement of T4 and T7 co-inoculations demonstrate T4 favoured over duration of phage competition. (A) Optical density (OD_{600}) measurements of two co-inoculations carried out in duplicate visualizing the drop in OD over time. 1 ml of supplementary *E coli* 600 cells were added to the co-inoculated cultures every ten minutes to replenish host cells. Un-inoculated control was monitored in parallel to ensure viability of host bacteria (B) Virus Like Particles (VLP) measurements derived from the flow cytometry data comparing the distinct population densities of T4 and T7 over the course of a co-inoculation experiment. Data obtained is representative of a single experiment. Replicates were not completed due to time constraints. Further experiments need to be completed to validate data.

represented by green fluorescence^{hi}, whereas the positive control for T7 (Fig. 4b) suggests this population is represented by green fluorescence^{low}. These results were consistent with genome sizes. Problems with our methods materialized when observing the negative control plot. Unexpected background fluorescence introduced challenges in differentiating T7 phage populations. These complications highlight the sensitivity of this method, as such, optimizing the preparation of the samples is required in order to minimize the amount of non-phage populations yielded in this assay. It is suspected that optimized experimental protocols would result in little to no background. To compensate for background fluorescence, the assumption was made that the frequency of background was constant throughout control and co-inoculated samples. This assumption allowed us to estimate T7 populations by eliminating the proportion of VLP's assumed to be background fluorescence. We assert this method of compensation is sufficient to quantify and discuss the data obtained from our co-inoculation samples. Our data is limited by our assumptions, and phage quantification should be taken as estimates. Regardless, we have demonstrated that T4 and T7 phage populations can be differentiated using flow cytometry, and optimized flow cytometry experiments with proper sample preparation would eliminate aforementioned limitations.

Despite difficulties in distinguishing our T7 phage populations, we were able to visualize the progression of a T4/T7 co-inoculation using VLP/ml and OD_{600} of infected cultures. OD_{600} measurements demonstrate bacteria density during co-inoculation experiments to mirror closely to that observed in T4 mono-inoculations. This could suggest T4 phage populations to be dictating the rate of cell lysis in a co-inoculated culture. It has been demonstrated that T4 phages are able to inhibit cell lysis in a competitive environment (12). By occupying host cells for longer periods, resources become inaccessible to competing T7 phages. Chan *et al.* had previously demonstrated that T4 phages out compete T7 during the absorption phase, in other words T4 are able to attach to the host cell at a faster rate than T7, supporting the argument that T4 are able to rapidly sequester the host cells for self. Our data display equal T4 and T7 concentrations (VLP/ml) at t=0, as expected, further supporting the sensitivity of flow cytometry over the DAOA in detecting the true ratio of phage without confounding variable of second infection event of indicator bacteria (Fig. 5b). at t=20, T4 concentrations were notably higher than T7 concentrations, supporting the theory that T4 bacteriophage may dominate early in co-inoculation by rapidly attaching and sequestering host cell resources. At t=60, a substantial drop in T4 and T7 concentrations was observed. This drop correlates with the OD_{600} plateau recorded at this time point during co and T4 mono-inoculations (Fig. 3, Fig. 7). It could be suggested this represents a secondary absorption phase for T4 bacteriophage. However, due to the sizable drop in both phage populations, it maybe suggested the results of this sample may have been skewed by experimental error. Time point t=80 and onwards display an exponential increase in T4 phage concentration, suggesting that T4 ultimately out competes T7 in this preliminary co-inoculation experiment. The addition of supplementary *E. coli* cells was essential to observe this competitive interaction. By supplementing the growing population of phage particles with the limiting resource, an extended visualization of competition could be recorded. It is possible that without supplementation complete lysis could have occurred prematurely. This

strategy allowed us to observe the exponential increase in T4 relative to T7 at time points 100 and 120 (Fig. 5b). This dominant result may be due to adaptations such as adsorption rate and lysis inhibition. Further replicates of this experiment with optimal flow cytometry sample preparation are necessary to substantiate this theory.

In terms of functional relevance, it is important to note that flow cytometry measures the amount of virus genomes present in the sample whereas the DAOA measures plaque forming units. As such, it is possible that flow cytometry will be counting viral particles that are not capable of infecting bacteria. For the purposes of monitoring competition between phage, this could lead to a distorted perception of the competitive environment. This caveat can be addressed through taking samples in duplicate. One sample follows the flow cytometry protocol as described in the methods section, while the other is mixed with indicator bacteria. This sample will be incubated in order to allow for adsorption, after which time the sample would be pelleted, and the supernatant, containing virus particles unable to infect the bacteria, would be extracted and subjected to flow cytometry. This would allow for an estimation of the number of inert virus particles at each sampling time point. At this time, it is challenging for us to comment on the precision of this assay, as only one replicate was performed due to time constraints. However, flow cytometry is currently the standard method used for enumerating phage. Moreover, with flow cytometry being a high throughput assay, it is much more efficient in its ability to analyze phage proportions in comparison to the DAOA, being a highly laborious method requiring multiple plates in varying dilutions for each time point.

The effectiveness of both the DAOA and flow cytometry in quantifying bacteriophage populations is dependent on the ability to confirm the purity and identity of the phage stocks being used and the resulting samples generated from them. Therefore, over the course of this study it was imperative to routinely assess our phage stocks for purity using PCR. Though we were able to demonstrate that our T4 and T7 mono-inoculation samples were free of cross contamination, the P^u lysate was propagated and did not amplify with any of our primer sets despite its ability to generate plaques in the DAOA. Through the use of flow cytometry and the DAOA, it was determined that P^u produced T7-like plaques and had a genome size much smaller than T4 despite its isolation from a commercial stock of T4 phage (Fig. 3c, 4d). It is possible that this phage is a T7 variant with capsid sequence mutations limiting the effectiveness of our primers for amplification, however it is important to consider the possibility of P^u being an environmental contaminant of another species entirely. This setback outlines a distinct limitation in the approach used to assess purity and identity of phage stocks in this and previous studies. By only screening for the three phage species previously used in this lab (ϕX174, T7, and T4), a positive confirmation of phage stock purity cannot be determined. Though the larger range of T7-like plaques of P^u were never observed in T4 phage isolates over the course of this study, it cannot be ruled out that P^u could have been present in T7 samples over the course of our experiments. Further investigation will be required to assess any impact P^u may have had on data collected in both the DAOA assay and flow cytometry.

In conclusion, despite the high degree of functional relevance of the DAOA with respect to lytic cycles in mono-inoculations, the inability to reliably differentiate between populations of T4 and T7 using this assay make it a poor candidate for studying competition dynamics. In contrast, the precision, differentiation of two phages, and efficiency of flow cytometry deem the method a favourable alternative for future studies. These findings will allow for more rigorous bacteriophage experimentation in future Microbiology 421/447 projects.

FUTURE DIRECTIONS

Phage stock titration in this study was performed using DAOA (providing concentrations in units of pfu/ml) and carried forward for calculations in preparing infections for analysis using flow cytometry. As flow cytometric enumeration detects phages in VLP/ml, our MOI calculation was incongruent with the method as apparent in not detecting equal ratios of T4 and T7 VLPs at t = 0 despite using identical MOIs (Fig. 5b). Future experiments can be optimized through re-titration of phage stock using flow cytometric enumeration and experimental determination of the ratio pfu/VLP for each phage such that results from both methods may be translated to the other for mathematical purposes. Further, including sufficient replicates of time course mono-inoculations and co-inoculations in varying MOI ratios may provide insight into the competitive relationship through the Lotka-Volterra model of interspecific competition (13). The Lotka-Volterra equation approximates logistic population dynamics of competing species, accounting for resource availability and species interaction (SFig. 2). The information of single phage population characteristics necessary to formulate a model using the equation can be derived experimentally from mono-inoculations and co-inoculations.

The unidentified phage contamination present in these experiments requires further pursuit of identification, which may be achieved through sequencing and PCR amplification through the use of degenerate primers targeting capsid genes (14). Efforts to prevent further contamination and identify sources of contaminating phage populations may be optimized in the process for future experiments using either methods outlined in this paper for phage enumeration.

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