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Growth phase of host *Escherichia coli* MG1655 influences T4 and T7 bacteriophage replication patterns during coinfection.

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SUMMARY T4 and T7 bacteriophages are proposed to compete during coinfection of *Escherichia coli*, impacting their replication relative to monoinfection conditions. During monoinfection of *E. coli*, T4 has been suggested to replicate optimally in exponential phase cells, while T7 in stationary phase cells. Our study investigates whether similar replication patterns will be observed during the coinfection of T4 and T7 in *E. coli* MG1655 growing in either the early exponential and stationary growth phases. Phage replication was followed post-infection by quantitative real-time Polymerase Chain Reaction over six hour period. During coinfection, we observed that T4 achieves higher relative copy number in the early exponential phase. Although both phages have reduced copy number in the stationary phase, compared to exponential phase, T7 exhibits higher copy numbers compared to T4, suggesting that it replicates more effectively in later phases of growth. Our results indicate that competitive interactions between T4 and T7 bacteriophages are indeed impacted by the growth phase of the host cells.

INTRODUCTION

As the global antibiotic resistance crisis becomes increasingly urgent, scientists and clinicians are seeking new treatments to fight bacterial pathogens resistant to multiple frontline antibiotics. One re-emerging remedy is phage therapy, in which bacteria-specific viruses, or bacteriophages, are used to combat bacterial infections (1). Phage therapy was first attempted in 1919, but the lack of understanding of phage mechanisms and insufficient purity of phage preparations prevented wider application (2). Phage therapy has recently gained momentum with the recent creation of an online database called the Phage Directory. This database contains a list of phages and their associated drug resistant bacterial targets (3). However, a greater understanding of phage biology is needed in order to inform the development of clinical applications.

T4 and T7 are double-stranded DNA bacteriophages that infect gram-negative bacteria such as *Escherichia coli*. Staples of classical phage and molecular biology, T4 and T7 have been extensively studied, which allows for more focused studies and in depth analysis. The two phages share similarities, such as gaining entry into a host cell by using their tail fibres to bind lipopolysaccharides on the outer membrane of the bacterium (4,5), and replicating through a lytic life cycle in which host cells are lysed to release new phage particles (4,5).

However, T4 and T7 phage show different infection characteristics during specific growth phases of *E. coli*. T4 has been shown to exhibit increased numbers of plaques when inoculated into *E. coli* cultures in early exponential phase (6), while T7 produces higher number of plaques in stationary phase cultures (7). Schrader *et al*. found that the burst size of T7, which is the number of phages produced per infected bacterium, is nine times larger in the stationary phase than in the early exponential phase (8). The same study stated that T4 does not appear to replicate in starved cells in stationary phase (8).

While the biology of these two phages have been extensively characterized, few studies have investigated the impact of competitive coinfection on model phages such as T4 and T7. One study, conducted by Chan *et al.* found that during coinfection of T4 and T7 phages in *E. coli* C600, T4 exhibited greater plaque formation than T7 in early exponential phase cells (6). In contrast, after infecting *E. coli* B23 with both phages, Nguyen *et al*. concluded that even though the replication rate for both phages decreased, neither phage "outcompeted" the other (9). These conflicting and inconclusive results beg for further investigation and hint at the

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complexity of phage interactions. Understanding this complexity will be key to developing effective phage therapies in clinical setting.

Herein, we investigated phage replication during coinfection of T4 and T7. Differential phage replication was studied as a function of host growth phase. Quantitative real-time polymerase chain reaction (qPCR) was used to accurately quantify phage replication in a temporal manner. Complete characterization of phage replication in various infection conditions will help contribute to the development of effective phage therapies.

METHODS AND MATERIALS

Strains and growth conditions. *E. coli* MG1655 and bacteriophages T4 and T7 were obtained from the Microbiology 421 culture collection from the Department of Microbiology and Immunology, University of British Columbia. The pUC19ΔSKM plasmid was kindly provided by Dr. John Nomellini. Plasmids were isolated using Bio Basic's EZ-10 Spin Column protocol as per manufacturer's protocol. Plasmid DNA was quantified by absorption using a NanoDrop 2000. *E. coli* were routinely cultivated at 37°C in Luria-Bertani (LB) Media as described in Sezonov *et al*. (10). 1.5% (w/v) agar plates were made from LB media.

***E. coli* MG1655 growth curve.** 400 µL of overnight *E. coli* culture was added to 40 mL of prewarmed LB media in 1:100 ratio and incubated at 37°C while shaking at 200 rpm. The optical density at 600 nm (OD_{600}) was monitored every 20 minutes using a Beckman spectrophotometer until exponential phase was reached, then every 40 minutes until stationary phase was reached. The cells were determined to be in stationary phase when three measurements in a row showed minimal changes in OD_{600}.

Propagation of T4 and T7 bacteriophages. Adapted from Pelzek *et al.* (11). T4 and T7 bacteriophages were amplified by infecting *E. coli* cells at OD_{600} of 1.0 with 1 mL of phage stock in LB media. The infected cells were incubated at 37°C while shaking at 200 rpm until clearing or reduced turbidity was observed. 1% v/v chloroform was added to release phage from the bacterial cells, and then the cultures were centrifuged for 10 minutes at 2,750 rcf at 4°C. Supernatant was gently collected and stored in 15 mL Falcon tubes at 4°C.

Double agar overlay plaque assays. Adapted from Pelzek *et al.* (11). 1.5% (w/v) LB agar was used for the underlay, and 0.75% (w/v) LB agar for the overlay. 100 µl of *E. coli* at OD_{600} 0.5 and 100 µl of a desired dilution (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-9}, 10^{-10}) of T4 or T7 phage were mixed gently, and then incubated at 37°C on a shaking platform (200 rpm) for 10 minutes. The mixture was added into 3 mL of the 50°C overlay agar, mixed gently, and poured over top of the underlay agar plate. Plates were incubated at 37°C overnight and plaques were quantified.

Mono- and coinfection of T4 and T7 in *E. coli*. 300 µL of overnight culture cells was inoculated into 30 mL of pre-warmed LB until the desired growth phase, as indicated by OD_{600}, was reached. The cells were split into three 10 mL volumes, and then infected with either T4 only, T7 only or both T4 and T7 at multiplicity of infection (MOI) of 3. The T4 only and T7 only infections were considered the "monoinfection" conditions. Cells infected with both phages were considered the "coinfection" conditions. From each tube, 21 x 150 µL (seven time points in triplicate) were aliquoted into a 96-well plate and incubated at 37°C. At various time intervals following the initial infection (0 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 24 hours), the OD_{600} values were used to monitor cell lysis. 1.5 µL chloroform (1% v/v) was used to lyse the infected cells and halt viral replication in 3 selected wells at each time point. Lysed samples were collected and stored at -70°C for later qPCR experiments.

PCR and agarose gel electrophoresis to confirm primer efficacy and specificity for use in qPCR. NCBI BLAST was used to check primer specificity *in silico*. To further ensure that primers would not form homo- or heterodimers and would only amplify their intended target, multiple primer combinations with or without template were tested. T4 and T7 primers were obtained from Wachtel *et al.* (12). The T4 primers were designed specific to the *gp23* region

Table 1 – Target genes and corresponding primer sequences

Gene	Sequence (5' - 3')	Size (bp)
T4 *gp23*	F: GCC ATT ACT GGA AGG TGA AGG R: TTG GGT GGA ATG CTT CTT TAG	398
T4 *gp23**	F: GTA TGG TAC GTC GTG CTA TTC R: TTG GGT GGA ATG CTT CTT TAG	150
T7 *gp10a**	F: CGA GGG CTT AGG TAC TGC R: GGT GAG GTG CGG AAC TTC	295
pUC19ΔSKM *ori**	F: CTA CAT ACC TCG CTC TGC TAA TC R: CAC GCT GTA GGT ATC TCA GTT C	189

**Used for qPCR analysis*

(accession number AF158101), T7 primers bind to the *gp10a* region (accession number V01146). The pUC19ΔSKM primers were designed to the *ori* region (accession number L09137), as shown in Table 1. The primers were ordered from Integrated DNA Technologies and used at a final concentration of 0.2 µM. The PCR reaction consisted of 0.5 µl undiluted T4 or T7 stock, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.2 µM of each primer pair (forward and reverse), 0.10 µl Platinum™ Taq DNA polymerase, and 1X reaction buffer in a final reaction volume of 25 µl with distilled water. The Bio-Rad T100™ Thermal Cycler was programmed to include a 10-minute initial denaturation step at 95°C followed by 33 cycles including: denaturation for 30 seconds at 95°C, annealing for 30 seconds at 50°C, and extension for 30 seconds at 72°C. All PCR products were run on a 2% agarose gel in 1X TBE buffer at 110V for 40 minutes. Bands were visualized using Invitrogen's SYBR™ Safe DNA Gel Stain and the Gel Doc UV imager. Desired amplification of pUC19ΔSKM, T4 and T7 target sequences using their corresponding primers was observed. There were strong bands at approximately 200 bp, 400 bp and 300 bp, which corresponded to amplification of the pUC19ΔSKM origin of replication, T4 *gp23* gene and T7 *gp10a* gene respectively (data not shown).

Generation of T4, T7 and pUC19ΔSKM standard curves using qPCR. Serial, tenfold dilutions from 10^0 to 10^{-6} of T4, T7 or pUC19ΔSKM sample were run in triplicate on a Bio-Rad T100™ Thermal Cycler. The same primer sets from PCR were used for both T7 and pUC19ΔSKM, while the T4 primers were designed to amplify a smaller region (150bp) of the T4 *gp23* gene, as indicated by the asterisk in Table 1. qPCR primers were used at final concentrations of 0.1µM. 0.5 µL of template DNA sample was added to 12 µL of 2X SYBR® Green Master Mix, 2.4 µL each of corresponding forward and reverse primers and sterile dH_2O to a final volume of 24 µL that was manually loaded onto a 96-well qPCR plate. The cycle was programmed to include a 10-minute initial denaturation step at 95°C followed by 39 cycles including: denaturation for 15 seconds at 95°C, and annealing for 30 seconds at 50°C. The Cq values for the standard samples were plotted against the corresponding log DNA phage dilution (Figure S1). The pUC19ΔSKM standard curve acted as our external control and its Cq values were used to normalize the T4 and T7 Cq values. T4 and T7 primers were seen to have similar efficiencies relative to pUC19ΔSKM external control primers (Figure S2A,B) . The standard curves were used to convert the Cq values obtained from experimental samples into DNA copy number. Specificity of each amplicon was determined by melt curve analysis from 65°C to 95°C (Figure S3).

Determination of relative phage DNA copy number from coinfection cultures. Frozen samples collected from the infection experiments were thawed and boiled for 5 minutes in 95°C using a heating block. The tubes were left to cool, and centrifuged using an Eppendorf™ 5424 Microcentrifuge at 13,000 x g for 1 minute. The same T4, T7 and pUC19ΔSKM primer

sets were used, as described under the standard curve generation section. The following reagents were combined into each qPCR well of a 96-well plate: 12 µL of 2X SYBR® Green Master Mix, 1 µL each of forward and reverse primers, 0.5 µL of the template DNA and sterile dH_2O to a final volume of 24 µL. The same cycle program described under the standard curve generation section was used. Melt curve analysis from 65°C - 95°C was also applied. Data was collected and analyzed using the Bio-Rad CFX Manager™ Software and GraphPad Prism 7.0. The Cq values of the experimental samples were compared to the slope of the standard curve in order to obtain the phage DNA copy number.

Each qPCR reaction that amplified either T4 or T7 bacteriophage had a corresponding reaction that amplified the pUC19ΔSKM DNA to determine variation between samples. For each replicate within the sample condition (specific growth phase, time point, infection condition), the copy number of pUC19ΔSKM, T4 bacteriophage, or T7 bacteriophage was determined from the Cq value using their respective regression equations generated from the standard curves (Figure S1A-B). The copy number of T4 or T7 was then multiplied by its respective dilution factor (1/100 for early and 1/500 for stationary phase).

Normalization of pUC19ΔSKM within replicates. Since the technical replicates of each sample should theoretically have the same copy number of pUC19ΔSKM, a correction factor for the copy number of pUC19ΔSKM was applied by dividing the mean copy number of replicate 1 by the mean copy number of replicate 2. The copy number of pUC19ΔSKM at each time point in replicate 2 was then multiplied by the correction factor to result in identical mean copy numbers, while still accounting for the variation between samples. The result was a normalized pUC19ΔSKM copy number between replicates for each time point. The copy number of the phage at each corresponding time point was also divided by this normalized value to achieve a "relative copy number" of each phage.

Normalization of pUC19ΔSKM between growth phases. To compare relative copy number of each phage across different growth conditions, the pUC19ΔSKM copy numbers were normalized between the early exponential phase and stationary phase at each time point for each infection condition. The mean pUC19ΔSKM copy number of stationary phase was divided by the mean pUC19ΔSKM copy number of early exponential phase to create a correction factor. This was repeated for T4 monoinfection, T4 coinfection, T7 monoinfection, and T7 coinfection samples. The normalized pUC19ΔSKM copy number of early exponential phase for each infection condition was multiplied by the respective correction factor to generate a pUC19ΔSKM copy number that was normalized within the replicates as well as to the stationary phase sample of same infection condition. This new normalized pUC19ΔSKM copy number was used to compare T4 or T7 relative copy numbers between samples of different growth phases in Figures 5 and 6.

Normalization of pUC19ΔSKM between infection conditions. To compare relative copy number of each phage across different infection conditions, the pUC19ΔSKM copy numbers were normalized between T4 and T7 at each time point for each growth phase. The mean pUC19ΔSKM copy number of T7 was divided by the mean pUC19ΔSKM copy number of T4 to create a correction factor. This was repeated for T4 monoinfection in early exponential phase and stationary phase and was only required for T4 monoinfection condition because the coinfection conditions already used a common pUC19ΔSKM sample. The normalized pUC19ΔSKM copy number of T4 monoinfection in each growth phase was multiplied by the respective correction factor to generate a pUC19ΔSKM copy number that was normalized within the replicates as well as to the T7 sample of the same growth phase condition. This new normalized pUC19ΔSKM copy number was used to compare T4 or T7 relative copy numbers between infection conditions within each growth phase in Figure 3C and Figure 4C.

Statistics. Significance between samples was determined by two-way ANOVA analysis, confidence intervals were determined by linear regression analysis using GraphPad 7.0.

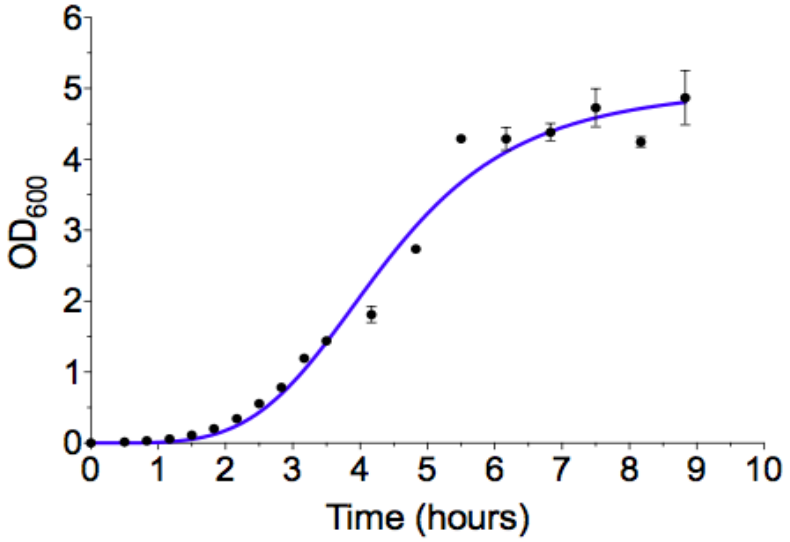

FIG. 1 Growth curve of *E. coli* MG1655 in LB media at 37°C at 200 rpm

RESULTS

A growth curve of *E. coli* MG1655 was established. In order to determine the growth phase of *E. coli* MG1655 at different OD_{600} measurements, LB media was inoculated with an overnight *E. coli* starter culture and OD_{600} was measured at 20 minute intervals. The growth curve in Figure 1 shows that early exponential phase (OD_{600} 0.5) was reached after approximately 2.5 hours of growth, while stationary phase (OD_{600} 4.5) was reached after approximately 8 hours of growth. This curve was used as a reference to carry out later growth phase-dependent phage infection experiments.

T4 and T7 phage were propagated and titre determined. Double agar overlay plaque assay was used to determine the concentration of each bacteriophage working stock that had been propagated in *E. coli*. Titer was calculated as plaque forming units (PFU) per mL. Plates with 30 to 300 plaques each were counted and averaged, yielding final values of 3.63×10^{10} PFU/mL for the T4 working stock and 4.53×10^{10} PFU/mL for the T7 stock. Plaques were observed to be of uniform size and shape for each phage, indicating that no cross-contamination had occurred between the phage lysates (Figure S4). Purity of each phage stock was later confirmed by PCR.

***E. coli* MG1655 cells in early exponential phase lyse at a faster rate in both coinfection and T7 monoinfection conditions than in T4 monoinfection condition**. To assess the differences in replication patterns between mono- and coinfection conditions at the early exponential growth phase, we generated a lysis curve by infecting *E. coli* at an OD_{600} of 0.5 with either T4, T7, or both phages. At various time points following the initial infection, we monitored OD_{600} readings and collected phage-infected samples at each time point for later qPCR analysis. As a control, OD_{600} readings of wells containing only LB were taken as a means of comparison and measure of complete cell lysis. Figure 2A shows the pattern of cell lysis via OD_{600} readings for all 3 conditions. The T7 monoinfection condition shows a nearly identical lysis trend as the coinfection condition, both dropping to an OD_{600} of approximately 0.08 by 2 hours post-infection. The T4 monoinfection condition exhibited a smaller initial drop in OD_{600} and although it continued to decline, it only reached an OD_{600} of approximately 0.25 by 6 hours post-infection. These results suggest that *E. coli* cells in early exponential phase may lyse at a faster rate during all conditions where T7 is present, compared to lower cell lysis in the T4 monoinfection condition.

***E. coli* MG1655 cells in stationary phase exhibit similar initial lysis rates in both monoinfection and coinfection conditions.** To assess the differences in replication patterns between mono- and coinfection conditions at the stationary growth phase, we generated a lysis curve by infecting *E. coli* at an OD of 4.97 with phages T4, T7, or both. Similar to the

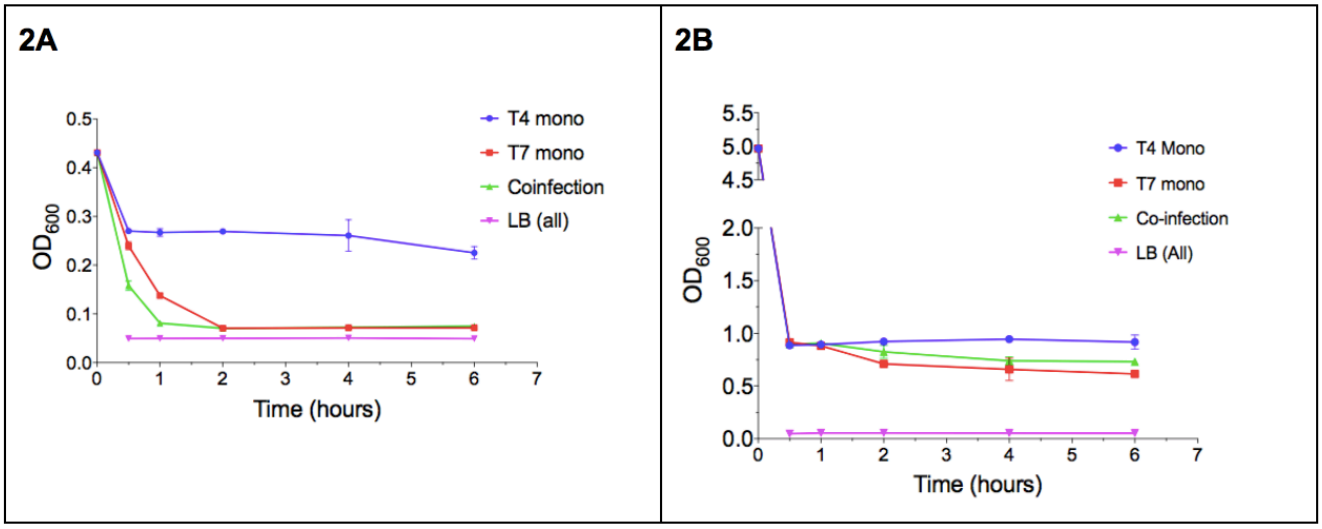

FIG. 2 *E. coli* lysis curves. (A) *E.coli* cells in early exponential phase growth with an OD_{600} of approximately 0.5 were infected with T4 bacteriophage, T7 bacteriophage, or both. OD_{600} of the infected wells and LB control wells was measured at various time points post-infection. **(B)** *E.coli* cells in stationary phase growth with an OD_{600} of approximately 5.0 were infected with T4 bacteriophage, T7 bacteriophage, or both. OD_{600} of the infected wells and LB control wells was measured at various time points post-infection.

early exponential phase infection, we collected samples at various time points for later qPCR analysis and simultaneously monitored OD_{600} readings. By 30 minutes post-infection, all samples had dropped in OD_{600} from 4.97 to approximately 0.8 (Figure 2B). After the 30 minute time-point, all 3 conditions slowed in their ability to clear the cell culture, with T4 monoinfection exhibiting a plateau at an OD_{600} of about 0.8 from 30 minutes post-infection onwards. T7 monoinfection and coinfection conditions continued to gradually decline in OD_{600} from approximately 0.8 at 1 hour post-infection to 0.6 and 0.7, respectively, at 6 hours post-infection. These results show that both monoinfection and coinfection of stationary phase cells exhibit similar initial patterns of lysis.

In early exponential phase, T4 appears to replicate more effectively than T7 in the coinfection condition, relative to their respective monoinfection conditions. In order to analyze the differences in phage replication patterns between mono- and coinfection conditions of *E. coli* cells infected during the early exponential growth phase, we used qPCR to measure relative T4 and T7 phage copy number at various time points following infection. The qPCR assay was chosen for its high sensitivity and efficiency relative to the double overlay plaque assay, which has also been used as a method of phage quantification (6). Both bacteriophage genomes consist of double-stranded DNA so this eliminated the need for a reverse transcriptase step prior to qPCR (4,5). A defined amount of pUC19ΔSKM was spiked into all samples as an external control and the resulting phage copy number was normalized to pUC19ΔSKM copy number within each condition. Based on the qPCR data, T4 exhibits higher relative copy numbers in coinfection conditions than in the monoinfection condition up to 4 hours post-infection (Figure 3A). In contrast, T7 exhibits lower relative copy numbers in coinfection conditions compared to its monoinfection condition up to 6 hours post-infection (Figure 3B). It should be noted that the data is not significant since only one technical replicate was used for T4 analysis (n=1). These preliminary results suggest that in early exponential phase, T4 in the coinfection condition may replicate more effectively than T7 compared to their respective monoinfection conditions.

In stationary phase, T4 appears to replicate less effectively than T7 in the coinfection condition, relative to their respective monoinfection conditions. In order to analyze the differences in phage replication patterns between mono- and coinfection conditions of *E. coli* cells infected during stationary phase, we used the same time-based qPCR approach as for early exponential phase analysis.The data suggests that both T4 and T7 in the coinfection

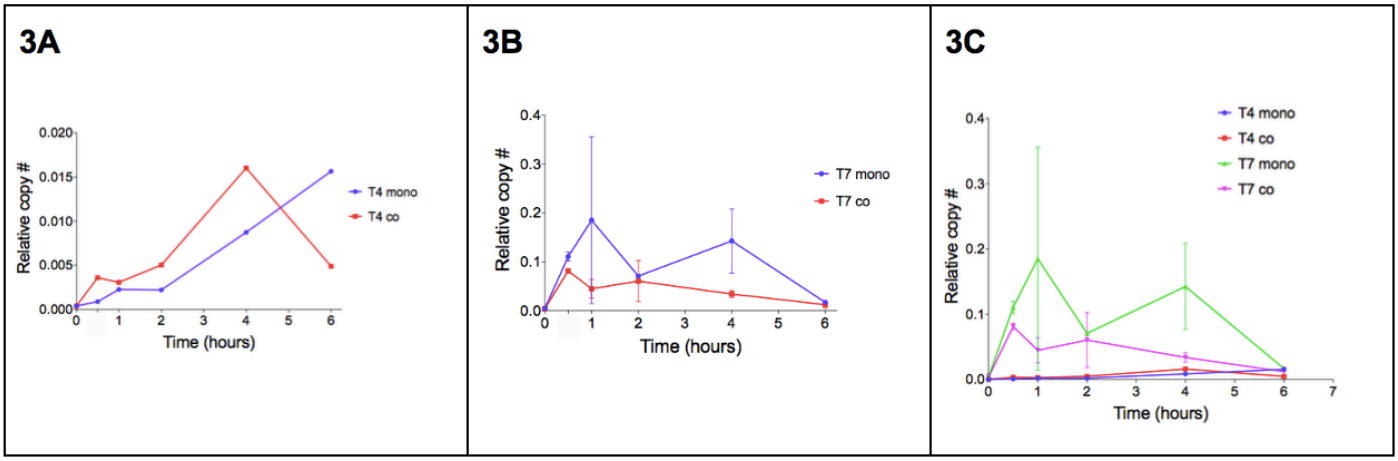

FIG. 3 Relative copy number of phage following infection of *E. coli* in early exponential phase. Phage copy numbers are normalized to pUC19ΔSKM copy numbers. **(A)** *E. coli* were infected with either T4 bacteriophage alone or were coinfected with both T4 and T7 bacteriophage. **(B)** *E. coli* were infected with either T7 bacteriophage alone or were coinfected with both T4 and T7 bacteriophage. **(C)** Merged figure of relative copy numbers of T4 and T7 bacteriophages following infection of *E. coli* at early exponential phase. Data was normalized between infection conditions to allow for direct comparison between T4 and T7.

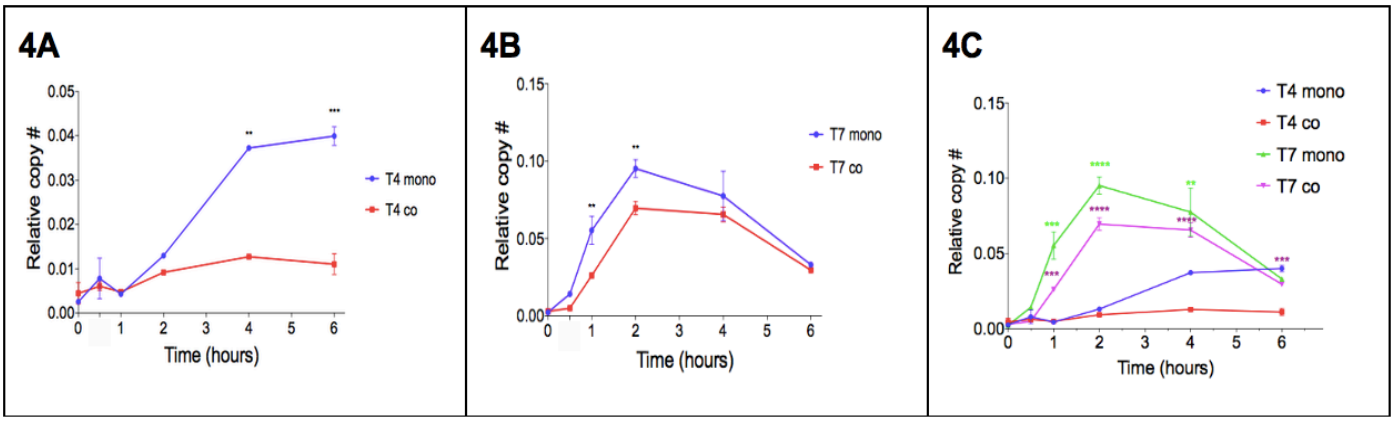

FIG. 4 Relative copy number of phage following infection of *E. coli* in stationary phase. Phage copy numbers are normalized to pUC19ΔSKM copy numbers. **(A)** *E. coli* were infected with either T4 bacteriophage alone or were coinfected with both T4 and T7 bacteriophage. P(4 hr) = 0.0028, and p(6 hr) = 0.0002. **(B)** *E. coli* were infected with T7 bacteriophage alone or were coinfected with both T4 and T7 bacteriophage. P(1 hr) = 0.002 and p(2 hr) = 0.0058). **(C)** Merged figure of relative copy numbers of T4 and T7 bacteriophages following infection of *E. coli* at stationary phase. Data was normalized between infection conditions to allow for direct comparison between T4 and T7. At 1 hour, 2 hours, and 4 hours post-infection, the T7 monoinfection condition compared to the T4 monoinfection condition has P-values of 0.001, $p < 0.0001$, and $p = 0.0052$, respectively. At 1 hour, 2 hours, 4 hours, and 6 hours post-infection, the T7 coinfection condition compared to the T4 coinfection condition has P-values of 0.004, $p < 0.0001$, $p < 0.0001$, and $p = 0.0002$, respectively.

condition exhibited lower relative copy number when compared to their respective monoinfection controls (Figure 4A,B). The T7 coinfection condition is significantly lower than its monoinfection control at 1 and 2 hours post-infection ($p = 0.002$ and $p = 0.0058$, respectively), and the T4 coinfection condition is significantly lower than its monoinfection control at 4 and 6 hours post-infection ($p = 0.0028$ and $p = 0.0002$, respectively). Despite both T4 and T7 experiencing a significant drop in copy number in coinfection conditions, T4 experienced a roughly 4-fold decrease in maximum copy number when compared with the monoinfection condition, whereas T7 experienced less than a 2-fold decrease in maximum copy number. These results show that T4 may replicate less effectively than T7 in coinfection conditions, relative to their respective monoinfection conditions.

T7 reaches higher relative copy numbers when cells are infected in stationary phase. Using the same data from the phase-dependent *E.coli* qPCR experiments, we analyzed differences in T7 replication patterns between the two growth phases, early exponential or stationary phase, across the different infection conditions. Across all the conditions involving T7, the phage reached the highest copy number relative to pUC19ΔSKM when cells were mono- and coinfected in stationary phase. The peak copy number in the stationary condition is seen to reach a relative value of approximately 0.1 (Figure 5). Although T7 monoinfection copy number also reaches a peak of approximately 0.1 in early exponential phase at 1 hour, there is a higher standard deviation and a consistently lower relative copy number at all time points past 1 hour. Based on statistical analysis, T7 exhibits significantly higher copy numbers during coinfection in stationary phase compared to early exponential phase at 2, 4, and 6 hours post-infection ($p = 0.0003$, $p < 0.0001$, and $p = 0.0233$, respectively). These results indicate that T7 reaches higher relative copy numbers when cells are infected in stationary phase versus early exponential phase.

T4 reaches its highest relative copy number during monoinfection of stationary phase cells. Similar to the T7 phage analysis across all growth conditions, we analyzed conditions involving T4 phage across the two growth phases in order to assess differences in T4 replication patterns between early exponential and stationary phase infected cells. T4 reached the highest relative copy number when cells were monoinfected in the stationary growth phase, as seen in Figure 6. Relative copy number of T4 in this condition continued to increase 6 hours post-infection, reaching a relative copy number of approximately 0.04. In the early exponential monoinfection condition, T4 experienced a gradual increase in copy number but only reached a relative copy number of approximately 0.01 after 6 hours of infection. At 4- and 6-hours post-infection, relative copy number of T4 monoinfection condition at stationary phase is significantly higher than the T4 monoinfection condition at early exponential phase ($p = 0.0243$, and $p = 0.0171$, respectively). These results show that T4 reaches a higher relative copy number during monoinfection of cells in stationary phase when compared to all infection conditions involving T4.

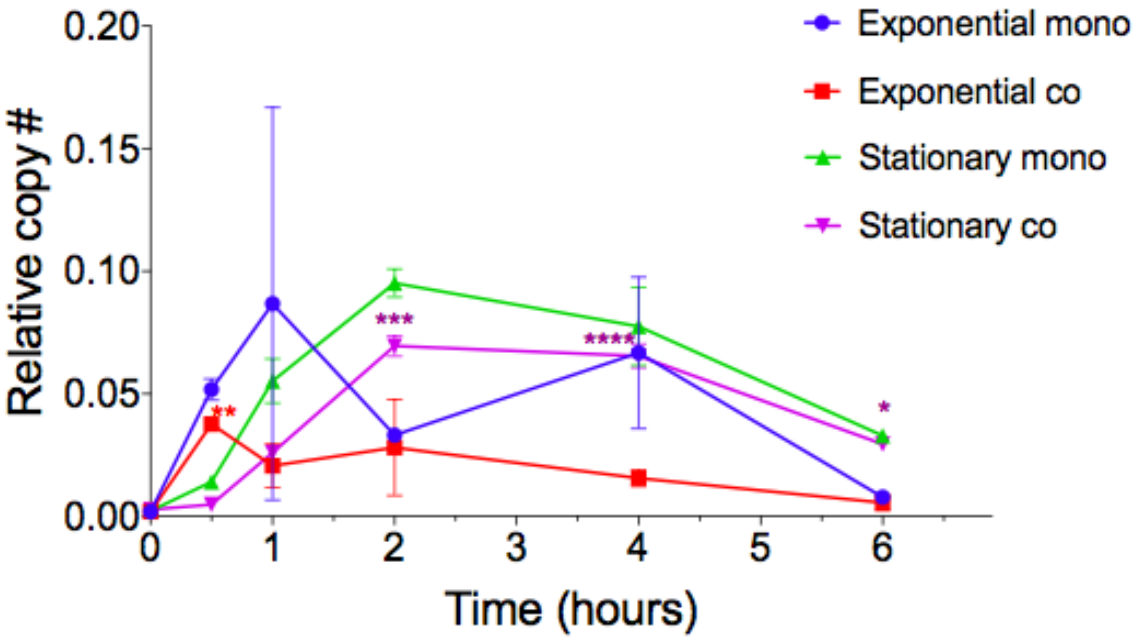

FIG. 5 Relative copy number of T7 bacteriophage following infection of *E. coli* in either stationary phase or early exponential phase. Phage copy numbers are normalized to pUC19ΔSKM copy numbers between sample replicates and between growth phage conditions as described in the materials and methods**.** At 0.5 hours post-infection, the T7 coinfection condition at early exponential phase compared to the T7 coinfection condition at stationary phase has a P-value of 0.0022. At 2 hours, 4 hours, and 6 hours post-infection, the T7 coinfection condition at stationary phase compared to the T7 coinfection condition at early exponential phase has P-values of 0.0003, $p < 0.0001$, and $p = 0.0233$, respectively.

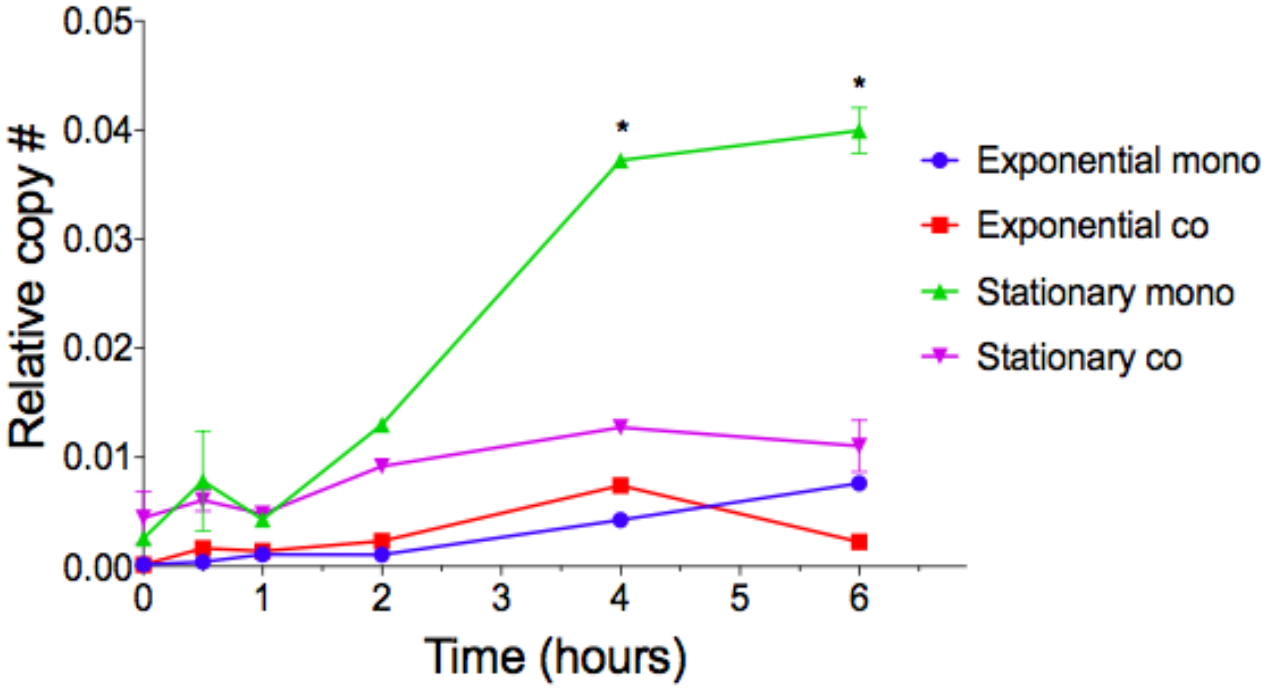

FIG. 6 Relative copy number of T4 bacteriophage following infection of *E. coli* in either stationary phase or early exponential phase. Phage copy numbers are normalized to pUC19ΔSKM copy numbers between sample replicates and between growth phage conditions as described in the materials and methods**.** At 4 hours and 6 hours post-infection, the T4 monoinfection condition at stationary phase compared to the T4 monoinfection condition at early exponential phase has P-values of 0.0243, and p = 0.0171, respectively.

T7 reaches a higher maximum copy number than T4 in all infection conditions. As an overall comparison of relative copy number between T4 and T7 phage, we assessed relative phage copy number between all mono- and coinfection conditions within each growth phase. When cells were infected in early exponential phase, T7 in both mono- and coinfection conditions exhibited higher relative copy numbers than their T4 counterparts (Figure 3C). When cells are infected in stationary phase, T7 exhibits significantly higher relative copy numbers than T4 in both mono- and coinfection conditions (Figure 4C). At 1, 2, and 4 hours post-infection, the T7 monoinfection condition has significantly higher relative copy numbers than the T4 monoinfection condition ($p = 0.001$, $p < 0.0001$, and $p = 0.0052$, respectively). At 1, 2, 4- and 6-hours post-infection, the T7 coinfection condition has significantly higher relative copy numbers than the T4 coinfection condition ($p = 0.004$, $p < 0.0001$, $p < 0.0001$, and $p = 0.0002$, respectively). The trend of the data indicates that across both growth phases and infection conditions, T7 consistently reaches a higher maximum relative copy number than T4 phage.

DISCUSSION

Previous studies have shown conflicting results regarding the nature of competition between bacteriophages T4 and T7 (6,9). This study explores the nature of interaction of these model phages during an *E.coli* coinfection model. More specifically, this study establishes that competition between these phages does affect their replication patterns in a manner dependent on the growth phase of the bacterial host.

In co-infection conditions, T4 appeared to replicate more effectively than T7 in early exponential *E. coli* cells and T7 appeared to replicate more effectively than T4 in stationary *E. coli* cells. In this context, the relative "effectiveness" of each phage's ability to replicate was defined in comparison to the monoinfection controls that were run at the same time. Comparison to a monoinfection control was deemed necessary due to differences in average burst size between the two phages (8). Coinfected T4 phage achieved a higher relative

maximum copy number compared to the monoinfected control in early exponential phase cells; however in stationary phase cells, both T4 and T7 were observed to exhibit lower maximum copy numbers in the coinfection condition than their respective monoinfection controls. Overall, T7 was considered to replicate more effectively than T4 in coinfection conditions since T7 exhibited a smaller decrease in maximum copy number relative to its monoinfection control, which is consistent with literature showing that T7 thrives in stationary phase cells (8).

The results indicate that the relative success of each virus to replicate effectively during competitive coinfection appears to be dependent on the growth phase of the infected cells. This suggests that the phage may use internal mechanisms to compete with each other following entry and co-infection of the host. These competitive mechanisms are likely influenced by a complex interplay of processes occuring in the cell at any given time. As a result, the transition of the cell into a stress response state could serve to favour of one phage over the other (13). Cells in stationary phase have been shown to display a stress response due to scarcity of nutrients, and therefore the resulting changes in gene expression could potentially give T7 a competitive advantage over T4 in stationary phase conditions, as is consistent with our results (13).

Interestingly, between the two growth phases, both T4 and T7 appeared to replicate optimally during monoinfection of cells in stationary phase compared to early exponential phase (Figure 5, 6). This contradicts some previous studies which have reported that T4 is unable to replicate in late exponential or starved stationary phase *E.coli* cells (7,8). Our lysis data provides further evidence for the enhanced ability of T4 to replicate in stationary phase cells, as *E. coli* cultures in stationary phase are observed to experience significant decrease in cell density (indicating cell lysis) following infection with T4 phage (Figure 2B). In contrast, T4 monoinfected cells in early exponential phase appear to lyse at a slower rate, showing smaller changes in cell density compared to stationary phase cells, and failing to completely lyse the cells (Figure 2A). Recent studies support this conclusion that T4 can replicate effectively in stationary phase, however, the question remains as to why T4 and T7 both undergo greater replication in stationary cells as opposed to the nutrient rich cells of early exponential phase (14,15).

It is possible that the reduced ability of T4 and T7 to replicate in early exponential phase cells stems from the reduced density of cells in this phase compared with stationary phase. With larger, more frequent gaps between cells and no mechanism for motility, the phage may not have as many opportunities to interact with and bind to their host cells. The fact that the cultures were not shaken during incubation with the phage may have further amplified this effect between the two growth conditions, thereby stunting the replication of the phage in the early exponential phase cells. In this scenario, the data would not be accurately reflecting the ability of the phage to replicate in the internal environment of the cells, but rather the fluid dynamic differences between the phases and the probabilities of interactions with host cells. If the equipment were available and time, it would therefore be beneficial to repeat this experiment with continuous shaking of the cultures to address that variable.

Another mechanism that may explain the differing replicative abilities of T4 and T7 phage within growth phases is the survival responses that phage are thought to exhibit under particular environmental conditions. It has been suggested that T4 infecting stationary phase *E.coli* cells can exhibit one of two responses: the hibernation response or the scavenger response (15). In hibernation mode, T4 pauses its replication cycle until more nutrients become available, whereas in scavenger mode, it continues to make as many particles as it can with the nutrients available (15). The mechanism behind the shift into either one of these modes is not well understood. It is possible that co-infection with T7 phage could trigger T4 to enter into hibernation mode, which would explain the low number of T4 phage we observe in co-infection conditions in stationary phase (Figure 4A). An alternate reasoning to explain the apparent enhanced replication of T7 relative to T4 in these conditions, could be that T7 has a more effective scavenger response that allows it to sequester nutrients for its own use more efficiently than T4 can.

It should be noted that our data from qPCR analysis has little statistical significance due to a low number of replicates. Only one technical replicate was performed for the early exponential phase T4 conditions, and two technical replicates for all other phage and growth

phase combinations. This limits our ability to draw concrete conclusions from the data, however, general trends can still be observed. For the most part, the trends in qPCR data are consistent with the cell density readings. For example, all cell cultures containing T7 exhibit more complete cell lysis than cultures containing T4 only (Figure 2A, 2B). This is reflected by the fact that T7 consistently had higher maximum relative copy number in all conditions and growth phases compared to T4 (Figure 3C, 4C) . This consistency with the lysis curves gives us more confidence in the qPCR analysis, since all cell lysis data was gathered in triplicate and shows minimal variability.

Limitations Three biological replicates were collected for each time point in each infection condition, however we only had the time to analyze one biological replicate of each. In addition, we ideally would have performed at least 2 technical replicates on each biological sample, but we had to forego the second technical replicate for all samples infected in early exponential phase due to project constraints. This lead to significant variability in our results and made it difficult to perform statistical analyses on those samples. If sufficient time and resources were allocated, we would be keen to improve the validity of our results by analyzing the remaining biological replicates with at least two technical replicates for each. The goal of this would be to increase confidence in the data and therefore allow us to draw more robust conclusions, including comparisons of numerical fold changes in relative copy number for every time point between the mono- and coinfection conditions.

Conclusions In conclusion, we employed a qPCR-based assay to analyze the effects of coinfection on T4 and T7 replication patterns in both early exponential and stationary growth phases of *E.coli* MG1655 cells. Although our findings are preliminary due to low replicate number, our results indicate that competitive interactions between T4 and T7 bacteriophages are impacted by the growth phase of the host cells. These results are consistent with the hypothesis that T4 replicates more effectively in early exponential coinfection conditions, and that T7 replicates more effectively in stationary phase coinfection conditions

Future Directions To further investigate the extent to which each T4 and T7 phage are affected by coinfection conditions, it would be interesting to perform the same time course experiments but to collect samples at shorter time intervals within the first 1-2 hours post-infection. This would allow for the generation of a more robust replication curve which could be used to determine the initial rate of viral replication. This could be used for a more in-depth analysis of the differences in phage behaviour between the growth phases or infection conditions. Mathematical modelling could be applied to the data to determine whether the variations in viral replication rate between the growth phases are a result of differential burst size, or rather a result of an increased or decreased lytic cycle time for each phage. Mathematical analysis could also be applied to determine an average burst size for T4 during stationary phase since, to our knowledge, this has not been previously characterized and could be useful for future modelling of viral replication patterns. It could also be valuable to replicate the experiment with different host organisms to observe whether the host strain changes the interaction between the viruses. Alternately, the nature of the interactions between additional strains of viruses could be explored in a similar manner.

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