Journal of Experimental Microbiology and Immunology (JEMI) Vol. 18: 162 – 166

T4 Bacteriophage May Not Inhibit Transcription of T7 Bacteriophage Genes rpol and gp10a During Co-infection of *Escherichia coli*

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When viruses simultaneously infect the same host cell, this can lead to an altered outcome of infection for the co-infecting pathogens. During the co-infection of *Escherichia coli* strain C600 with both T4 and T7 bacteriophage, T4 prevents the production of T7 progeny. It is unclear whether T4 inhibits the transcription of T7 genes as a means to achieve this outcome. Thus, the relative expression of early- and late-phase T7 genes, rpol and gp10a, respectively, as well as late-phase T4 gene gp23 were determined during co-infection of *E. coli* C600 with T4 and T7 phage. Infected cultures were sampled at 20 and 38 minutes post-infection to identify whether rpol, gp10a, and gp23 transcripts were detectable. Upon optimizing the primer selection, DNase treatment, and PCR conditions, PCR amplification of the the co-infection cDNA yielded both T4 and T7 amplicons. The presence of T7 rpol, T7 gp10a, and T4 gp23 transcripts at both time points from the co-infection suggested that T4 phage likely does not inhibit the transcription of T7 genes as a means of preventing the production of T7 progeny during co-infection of *E. coli.*

Viral co-infection is the commonly observed phenomenon of a single host being concurrently infected with multiple pathogens, presenting a unique opportunity for pathogen-pathogen interactions (1). In the case of viruses, this may be beneficial for all infecting phage, while in other cases weaker phage may be outcompeted (1). Certain viruses have evolved targeted mechanisms to inhibit other viruses present in the intracellular environment, such as the sequestration of host enzymes (2). Competition with other viruses may also occur via direct inhibition of essential viral replication processes, such as transcription (2). In the case of T4 bacteriophage co-infection with T7 bacteriophage - both exclusively lytic coliphages (viruses which infect *Escherichia coli*) with monocistronic linear double-stranded DNA (dsDNA) genomes - the proliferation of T7 bacteriophage is suppressed as a result of an unknown mechanism (3).

T4 bacteriophage have promoters that allow immediate early transcription of genes that recruit host proteins (e.g. RNA and DNA polymerases) for its own use, possibly preventing T7 from accessing the host's RNA polymerase for early gene transcription (4). This mechanism, however, may not be sufficient to completely inhibit viral replication of T7 bacteriophage (4). The T4 bacteriophage genome (169 kb) is over four times larger than that of T7 (40 kb). Further, T4 produces over 5 times more gene products, some of which are poorly understood and which may provide it with an advantage during co-infection (3, 4).

The bacteriophage life cycle may be separated into early, middle, and late phases of gene expression (4). At the early phase of viral replication, polymerases or polymerase-recruiting proteins are expressed to initiate rapid expression, while the later phases involve expression of genes encoding the proteins forming the capsid (4). T4 and T7 phage have comparable life cycle durations of about 30 minutes at 30^{o}C, with burst sizes of approximately 60 phage and 250 phage, respectively (5, 6)

The T4 bacteriophage Alc protein inhibits host transcription and thereby may also suppress T7 phage transcription and subsequent replication at a specific phase of its life cycle, which can be tracked by the expression of characteristic genes at early and late phase (2, 4). The T4 bacteriophage gene, gp23, encodes one of the three major capsid proteins for the virus (7). The T7 bacteriophage RNA polymerase, rpol, and major head capsid protein, gp10a, are transcribed during early and late phase, respectively (6, 7). It was observed in previous studies that during a co-infection of *E. coli* C600 by T4 and T7 bacteriophage, only viable T4 progeny was produced (3). Chan *et al.* also reported data suggesting that both T4 and T7 genome were present in the host cell during co-infection, implying that cellular exclusion was not a factor (3). The aim of this study was to investigate co-infection interactions between T4 and T7 bacteriophages in *E. coli* C600 at the level of transcription. The current literature on bacteriophage co-infection is scarce, in spite of the clinical significance of their *E. coli* hosts. The work herein developed a molecular approach to study this co-infection, and described the development of PCR primers and conditions used, as well as the feasibility of amplifying multiple phage genes simultaneously using a multiplex PCR protocol.

MATERIALS AND METHODS

Strains and growth conditions. Bacterial strain *Escherichia coli* C600 and T7 bacteriophage were obtained from the MICB 421 culture collection (Department of Microbiology and Immunology, University of British Columbia). T4 bacteriophage was obtained from Carolina Biological Supply Company (Burlington, North Carolina). *E. coli* cultures were grown at 30^{o}C in L-media broth, prepared according to Overby *et al.* (8). The T7 bacteriophage was amplified by infecting 2.4 x 10^{8} cell/ml *E. coli* C600 culture at a multiplicity of infection (MOI) of 0.1, followed by incubation for 4 h at 30°C and 180 rpm. Phage were harvested by centrifuging the lysates at 8000 x g for 10 min, after which the supernatant was collected and treated with 0.1% (v/v) chloroform

(Fisher Scientific, C298-500). The final T7 phage concentration was determined by a plaque assay. T4 bacteriophage amplified in *E. coli* B23 was obtained from MICB 421 lab colleagues, Khue-Tu Nguyen, Igor Tatarnikov, and Karen Simmons, used at a concentration of 1.0 x 10^{11} pfu/ml.

Plaque assays. The overlay agar plaque assay method described by Fortier and Moineau (9) was employed to enumerate phage and to confirm phage replication following mono- and co-infections using *E. coli* strain C600 as an indicator organism. L-media was used to make 1.5% (w/v) agar (Select Agar, Invitrogen, 30391-023) plates, which were overlaid with a 0.4% (w/v) soft-overlay agar. The phages were distinguished by differences in plaque morphology, T7 plaques being significantly larger in diameter than T4 plaques. Using sequential dilution, the T7 stock concentration was calculated to be 1.1 x 10^{10} pfu./ml.

Co-infection of phage in *E. coli* C600. An overnight culture was used to inoculate three Oakridge centrifuge tubes (Nalgene) with 7 ml of 3 x 10^8 cell/ml *E. coli* C600. Cultures were infected with either T4 (MOI 2.5), T7 (MOI 2.5), or both T4 and T7 (MOI 1.25 each) phage. After incubating the infected cultures for 5 min in a 30°C water bath at ~200 rpm, the cultures were centrifuged at 8000 x g for 5 min and the supernatant was discarded. Pellets containing infected cells were resuspended in 7 ml fresh media, and distributed into two 10 ml test tubes with 3 ml infected culture each, one for each time point. Tubes were incubated in the 30°C water bath at ~200 rpm after which a 3 ml sample was removed for RNA extraction after the elapse of 7 or 25 min (approximately 20 and 38 minutes post-infection). The phage for plaque assays were removed at the later time point, prior to RNA extraction.

RNA extraction, DNase treatment, and reverse transcription. Immediately after the samples were removed from the water bath, 100 ul stopping solution (95% EtOH, 5% phenol (Ultra-Pure Buffer-Saturated, Invitrogen, 15513-039)) was added and samples were then spun at 8000 x g for 3 min at 4°C, the supernatants were discarded, and the pellets were resuspended by adding 100 ul lysozyme solution (10 mg/ml lysozyme (Sigma, 235-747-3), 10 mM Tris-HCl (Sigma, T3253-500G), 0.1 mM EDTA (ThermoScientific, EN0525)). Next, 500 ul Trizol (Invitrogen, 15596-026) was added and samples were vortexed for 30 s, followed by the addition of 200 ul chloroform (Fisher Scientific, C298-500) to each sample and 2 min of thorough mixing. Samples were spun at 12,000 x g for 15 min at 4°C, then the transparent upper phases were transferred to new tubes. 800 ul of 70% EtOH was added, mixed well, and the entire volume was transferred to an RNeasy MiniSpin Column (Qiagen, 74104). The RNA was extracted as per the RNeasy MiniKit protocol, yielding 30 ul samples that were stored at -80°C. The extracts were treated with DNase I (Fermentas, EN0521) as per manufacturer's instructions. The RNA was reverse transcribed into cDNA using the Invitrogen SuperScript VILO cDNA Synthesis Kit (11754-050), following manufacturer's instructions.

Primer design and PCR. The primers used to amplify T4 and T7 gene segments were manufactured by Integrated DNA Technologies and designed using the Integrated DNA Technologies Oligo Analyzer 3.1 program (Table 1). The PCR reactions consisted of 0.5 µl sample, 0.2 mM dNTP mix (Thermo Scientific, R0192), 1.5 mM $MgCl_2$ (Fermentas), 0.5 µM of each primer pair, 0.5 U Taq Polymerase (Fermentas, EP0602), and 1X Reaction Buffer (Bio-Rad, 4206371) in a final reaction volume of 25 µl with distilled water. The reactions occurred in the Bio-Rad Gene Cycler Thermal Cycler (170-6700) with an initial denaturation of 93°C for 10 min, followed by 35 cycles of 93°C for 1 min, 54°C for 1 min, and 72°C for 1 min, and a final extension step at 72°C for 5 min. Samples were left overnight at room temp before being analyzed by agarose gel electrophoresis.

TABLE 1 Primers designed for amplifying fragments of T4 gp23, T7 rpol, and T7 gp10a. The letters f and r denote the forward and reverse primer, respectively.

Gene	Sequence (5' – 3')	Size (bp)
T4 gp23	f:GCCATTACTGGAAGGTGAAGG r:TTGGGTGGAATGCTTCTTTAG	398
T7 rpol	f:ACATGCTCTCTAAGGGTCTACTC r:GGAGTTCTTCACGCTCAATCG	517
T7 gp10a	f:CGAGGGCTTAGGTACTGC r:GGTGAGGTGCGGAACTTC	295

Multiple primer PCR testing at varying T4:T7 phage ratios. To test the limitations of multiple primer PCR reactions, a series of reactions with varying T4:T7 ratios of total sample phage were performed. The T4:T7 ratios chosen were 1:1, 8:1, 80:1, and 800:1 according to a previously published report of non-amplification in T4-dominant extractions (3). The amount of T4 phage was constant throughout the ratios, with the exception of a ten-fold dilution in the 1:1 ratio condition.

Agarose gel electrophoresis. PCR-amplified products were loaded with 6X DNA Loading Dye (Thermo Scientific, R0611) and resolved in 1.5% agarose gels (Ultrapure Agarose, Invitrogen) in 1X TAE buffer (40 mM Ultra-Pure Tris (Invitrogen, 15504-020), 20 mM glacial acetic acid (Acros, 64-19-7), 1 mM EDTA (EMD, EX0539-1), pH 8.5) at 100 V for 60-90 min with E-gel Low-Range Quantitative DNA Ladder (Invitrogen, 10068-013). Gels were stained in a 0.5 ug/ml ethidium bromide solution (lab stock) for 14 min and visualized with an AlphaImager MultiImage Light Cabinet (Fisher Scientific).

RESULTS

Primers specifically amplified gp23 gene from T4, as well as rpol and gp10a genes from T7. Primers were designed to amplify genes for early and late viral gene products. The two capsid genes (T4 gp23 and T7 gp10a) were chosen because of their high transcription levels, and T7 rpol (viral RNA polymerase) was chosen as it is the first gene to be transcribed and is the only T7 gene transcribed by the host RNAP. A plaque assay was done to confirm the identity and purity of the phage stocks. Mono-infections of the phage were analyzed by plaque morphology and amplification with the appropriate primers in PCR. T4 gp23, T7 rpol and T7 gp10a all showed specificity for the desired products after PCR testing against T4 and T7 phage in single primer set reactions (Fig 1). Lanes 2, 6 and 7 showed positive results, with the expected band sizes of approximately 400, 500, and 300 bp, respectively. Lanes 3, 4, and 5 showed negative results, indicating that the primers were genome specific. Under multiplex conditions, the gp10a primer set did not amplify the ~300 bp product from the T7 phage (lane 9; Fig 1). As conditions for a multiplex reaction needed further optimization in order to amplify T7 gp10a, the primers sets were used separately.

Band intensity after amplification with multiple primer sets was dependent on the relative sample concentrations of T4 and T7 phage. Prior to DNase treatment, the mono-infection and co-infection RNA extraction samples were amplified by PCR using T7 rpol and T4 gp23 primer sets to test for DNA contamination of

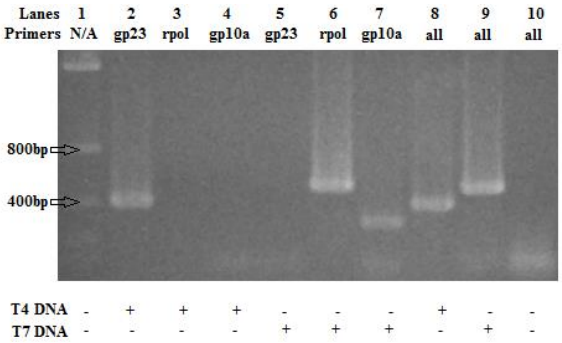

FIG 1 Verification of individual primer set specificities for fragments of the T4 gp23, T7 rpol, and T7 gp10a genes. The first lane contained the low MW DNA ladder.

the RNA samples (Fig 2). Products were seen in all lanes, indicating that the RNA was indeed contaminated with residual DNA (Lane 8 was a positive control using T7 DNA). For the T4 and T7 mono-infections at both time points (lanes 2, 5, 3, and 6), T4 gp23 and T7 rpol were amplified respectively, resulting in bands of comparable intensity. For the co-infection samples at both time points, only the T4 gene product gp23 was amplified, while none of the co-infection samples yielded visible bands for the T7 rpol fragment, suggesting that the T7 DNA was below the detection threshold. To investigate how the relative level of phage DNA affected amplification, a range of T4:T7 phage ratios were evaluated in a multiplex PCR reaction. At T4:T7 phage ratios of 1:1, 8:1, and 80:1, both gp23 and rpol genes were detected as ~400 and 500 bp bands, respectively (lanes 2, 3, and 4 of Fig 3). The intensity of the rpol bands decreased as the T4:T7 ratio increased, while the intensity of the gp23 bands remaining constant (Fig 3). The T7 (rpol) product was no longer detectable at the 800:1 ratio of T4:T7 phage (lane 4 of Fig 3).

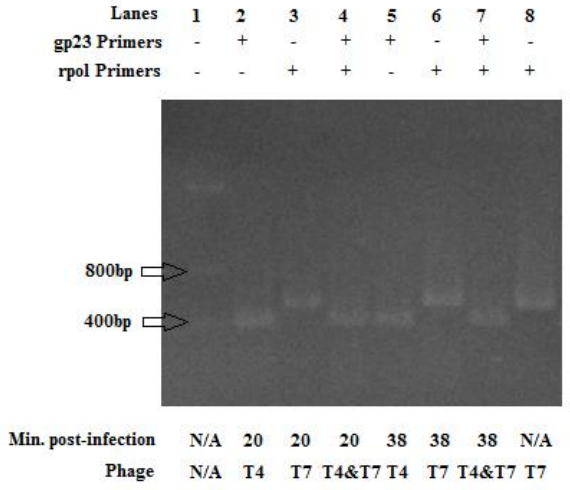

FIG 2 Testing RNA extractions for the presence of DNA contamination in samples from mono- and co- infections of *E. coli* C600 with T4 and/or T7 phage prior to DNase treatment or reverse transcription. The first lane contained the low MW DNA ladder.

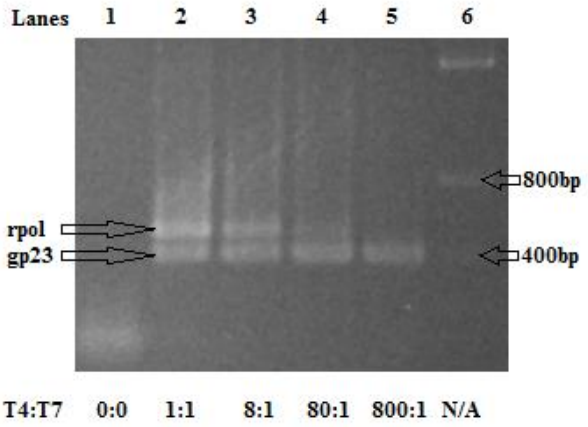

FIG 3 The effect of increasing T4:T7 phage ratios on multiple primer PCR amplification of T7 rpol, T7 gp10a, and T4 gp23. The last lane contained the low MW DNA ladder.

Isolation of DNA-free RNA from *E.coli* infected with T4 and T7 bacteriophage yielded variable results. The amplification of products from the RNA extraction samples indicated that the samples were contaminated with DNA, necessitating a DNase treatment to identify RNA transcripts exclusively (Fig 2). After DNase treatment, the RNA samples from the co-infection conditions at 7 min and 25 min were DNA-free, evidenced by the lack of PCR product (lanes 4 and 7; Fig 4). However, the DNase treatment was incomplete (product was amplified) for the RNA samples in lanes 2, 3, and 5. The DNase treatment gives variable results, and should be optimized for greater consistency in future experiments.

T4 and T7 transcripts were produced during a co-infection of *E. coli* C600. DNA-free RNA samples from the two co-infection timepoints were reverse transcribed to yield cDNA. PCR of the cDNA from the two co-infection time points showed bands in lanes 2-7 for T4 gp23, T7 rpol, and T7 gp10a at 20 and 38 minutes (Fig 5). This indicates that T4 and T7 mRNA transcripts were both present during the co-infection.

DISCUSSION

In order to elucidate the mechanism by which T4 bacteriophage prevents T7 phage production during co-infection, it was proposed that T4 might prevent T7 phage replication via transcriptional inhibition. Previous research implicated the T4 protein, Alc, in the termination of transcription of non-methylated cytosine-containing DNA by binding to host genome recognition sequences (10). While T4 has extensive 5-hydroxymethyl cytosine substitutions in its DNA, allowing continued transcription, the *E. coli* and T7 phage genomes have only a small percentage of methylated cytosine (11). Thus, one would expect that during a co-infection of T4 and T7 in *E. coli*, T4 would prevent the transcription of T7 genes, rpol and gp10a. To further investigate this, a PCR-based approach was developed to detect transcripts made during the co-

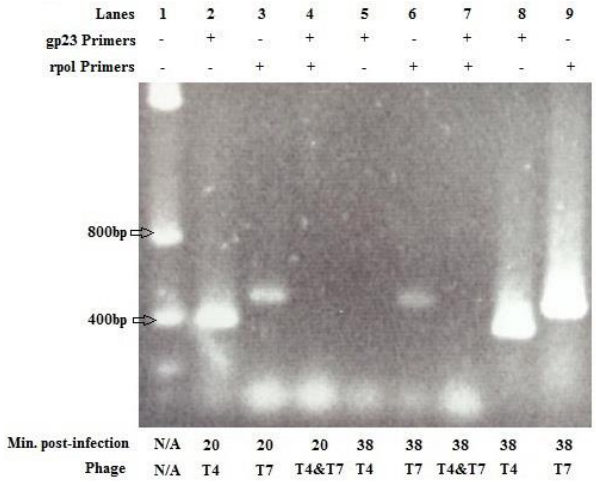

FIG 4 Testing RNA extractions for the presence of DNA contamination in co-infection samples post-DNase treatment. The first lane contained the low MW DNA.

infection. Primers were generated that amplified T4 gp23, T7 rpol, and T7 gp10a, with much time invested in optimizing PCR conditions.

Further optimization was required for the DNase treatment and reverse transcription in order to yield pure cDNA samples from the co-infections. The mono-infection samples contained residual DNA contamination even following multiple DNase treatments. In both time point samples from the co-infection, the T7 rpol was amplified from reverse transcribed cDNA, contrary to what was expected. This suggested that T4 does not use transcriptional inhibition as a mechanism to block the replication of T7 phage. This could result from an inability of T4 Alc from binding T7 DNA, prevented by the low amount of methylated cytosine and allowing continued transcription, or the absence of Alc binding sites on the T7 genome (11).

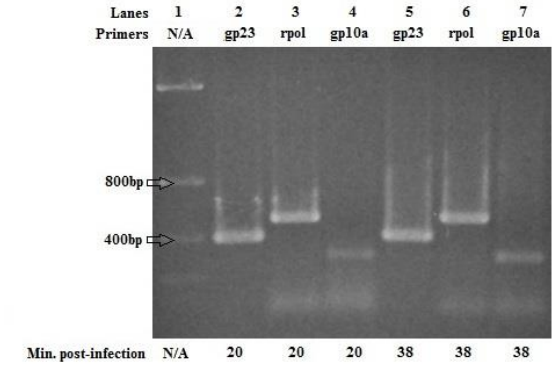

FIG 5 Amplified cDNA derived from co-infection RNA extractions subjected to DNase treatment and reverse transcription. The first lane contained the low MW DNA.

Prior to the DNase treatment, all of the extracted RNA samples were contaminated with DNA and although the T4 gene, gp23, was amplified in the T4 mono-infection samples and in the co-infection samples, only the T7 mono-infection samples yielded detectable rpol amplicons. The absence of the T7 gene, rpol, in the co-infection samples might suggest that T7 genomic DNA was not being replicated. In addition, a T4:T7 genomic DNA ratio greater than the 80:1 ratio was shown to be the upper limit for detectable amplification of the rpol fragment in Figure 3, and could account for the missing band.

To summarize, as it was previously reported that inhibition of T7 phage replication by T4 occurred during a co-infection, we proposed that it was global transcriptional inhibition of non-T4 genomes by T4 phage that was responsible for this effect. However, the presence of T7 RNA transcripts was observed during T4/T7 phage co-infection conditions. In conclusion, because our data showed that T4 was unable to prevent transcription of T7 genes during a co-infection of *E. coli* C600, it suggests that the inhibition of the T7 lifecycle by T4 occurs through another mechanism.

FUTURE DIRECTIONS

Future co-infection studies could investigate inhibition at the translational level by Western Blot and should include a plaque assay to better monitor the outcome of the co-infection and to confirm Chan *et al.*'s results (3). It may be valuable to investigate the expression of additional T7 genes. Also, the primers employed were able to qualitatively compare amounts of starting phage template in PCR with multiple primer sets, and could be used for qPCR applications investigating downregulation, rather than complete inhibition, of T7 gene products. Two amplicons, corresponding to the presence of rpol and gp23, were visible in multiple-primer reactions, but the optimization of conditions for a functional gp10a primer set in these PCR reactions would allow the simultaneous monitoring all three transcripts (or alternative targets, should they be identified). This would allow more efficient and accurate monitoring of co-infection progression.

ACKNOWLEDGEMENTS

We would like to thank Dr. William Ramey, Dr. David Oliver, Andrew Cameron, Lando Robillo, and the staff in the media room for their guidance and assistance. We would also like to thank our colleagues, Khue-Tu Nguyen, Igor Tatarnikov, and Karen Simmons, for providing us with T4 phage.

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