

Lysis Delay and Burst Shrinkage of Coliphage T7 by Deletion of Terminator T ϕ Reversed by Deletion of Early Genes

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ABSTRACT

Bacteriophage T7 terminator T ϕ is a class I intrinsic terminator coding for an RNA hairpin structure immediately followed by oligo(U), which has been extensively studied in terms of its transcription termination mechanism, but little is known about its physiological or regulatory functions. In this study, using a T7 mutant phage, where a 31-bp segment of T ϕ was deleted from the genome, we discovered that deletion of T ϕ from T7 reduces the phage burst size but delays lysis timing, both of which are disadvantageous for the phage. The burst downsizing could directly result from T ϕ deletion-caused upregulation of gene 17.5, coding for holin, among other T ϕ downstream genes, because infection of gp17.5-overproducing *Escherichia coli* by wild-type T7 phage showed similar burst downsizing. However, the lysis delay was not associated with cellular levels of holin or lysozyme or with rates of phage adsorption. Instead, when allowed to evolve spontaneously in five independent adaptation experiments, the T ϕ -lacking mutant phage, after 27 or 29 passages, recovered both burst size and lysis time reproducibly by deleting early genes 0.5, 0.6, and 0.7 of class I, among other mutations. Deletion of genes 0.5 to 0.7 from the T ϕ -lacking mutant phage decreased expression of several T ϕ downstream genes to levels similar to that of the wild-type phage. Accordingly, phage T7 lysis timing is associated with cellular levels of T ϕ downstream gene products. This suggests the involvement of unknown factor(s) besides the known lysis proteins, lysozyme and holin, and that T ϕ plays a role of optimizing burst size and lysis time during T7 infection.

IMPORTANCE

Bacteriophages are bacterium-infecting viruses. After producing numerous progenies inside bacteria, phages lyse bacteria using their lysis protein(s) to get out and start a new infection cycle. Normally, lysis is tightly controlled to ensure phage progenies are maximally produced and released at an optimal time. Here, we have discovered that phage T7, besides employing its known lysis proteins, additionally uses its transcription terminator T ϕ to guarantee the optimal lysis of the *E. coli* host. T ϕ , positioned in the middle of the T7 genome, must be inactivated at least partially to allow for transcription-driven translocation of T7 DNA into hosts and expression of T ϕ downstream but promoter-lacking genes. What role is played by T ϕ before inactivation? Without T ϕ , not only was lysis time delayed but also the number of progenies was reduced in this study. Furthermore, T7 can overcome T ϕ deletion by further deleting some genes, highlighting that a phage has multiple strategies for optimizing lysis.

Bacteriophage T7 is an obligate lytic *Escherichia coli* phage that has been extensively studied for more than 60 years (1). T7 RNA polymerase, the only RNA polymerase (gp1) in the T7 genome, is one of the best-characterized RNA polymerases, and its transcription mechanisms have been studied in detail (2–6). In the T7 genome, an intrinsic termination signal for T7 RNA polymerase, terminator T ϕ , is positioned between genes 10 and 11 at the late, class III region (Fig. 1A). Although the transcription termination mechanism of T7 RNA polymerase at terminator T ϕ has been rather well studied, there have been few reports thus far on the *in vivo* roles of this terminator.

Terminator T ϕ encodes a stable GC-rich RNA hairpin structure followed by a stretch of six U residues and the termination site G residue (Fig. 1B). Both the RNA secondary structure and primary sequence have been demonstrated to affect termination efficiency at T ϕ . *In vivo*, read-through at terminator T ϕ is necessary for the expression of T7 genes 11 and 12, as these two genes are located immediately downstream of T ϕ and do not have their own promoters. It has also been reported that formation of a hairpin structure derived from the T ϕ sequence at the 3' end of the gene 10 transcript helps increase stability of the transcript mRNA and thereby raises its expression level more than 2-fold (7). Additionally, a frameshift in gene 10 mRNA translation, necessary for pro-

duction of the minor capsid protein gp10B, also requires the T ϕ -derived hairpin structure at the 3' end of the mRNA, although failure of the frameshift shows little phenotypic effect on phage infection (8, 9). To our knowledge, there has been no report on the functions of terminator T ϕ during T7 phage infection at phenotypic levels.

The replication cycle of phage T7 typically consists of three phases: (i) searching for and adsorption to a host, followed by (ii) transfer of its linear DNA genome into a host cell and expressing phage genes, and completion when (iii) host cells are lysed to release newly assembled phage progenies. All three phases have been well studied at molecular levels (1). The *E. coli* receptor for phage T7 is lipopolysaccharide on the surface, which binds to the C-terminal end of T7 tail fiber protein (gp17). Genomic DNA is then translocated into the host cell in a transcription-dependent

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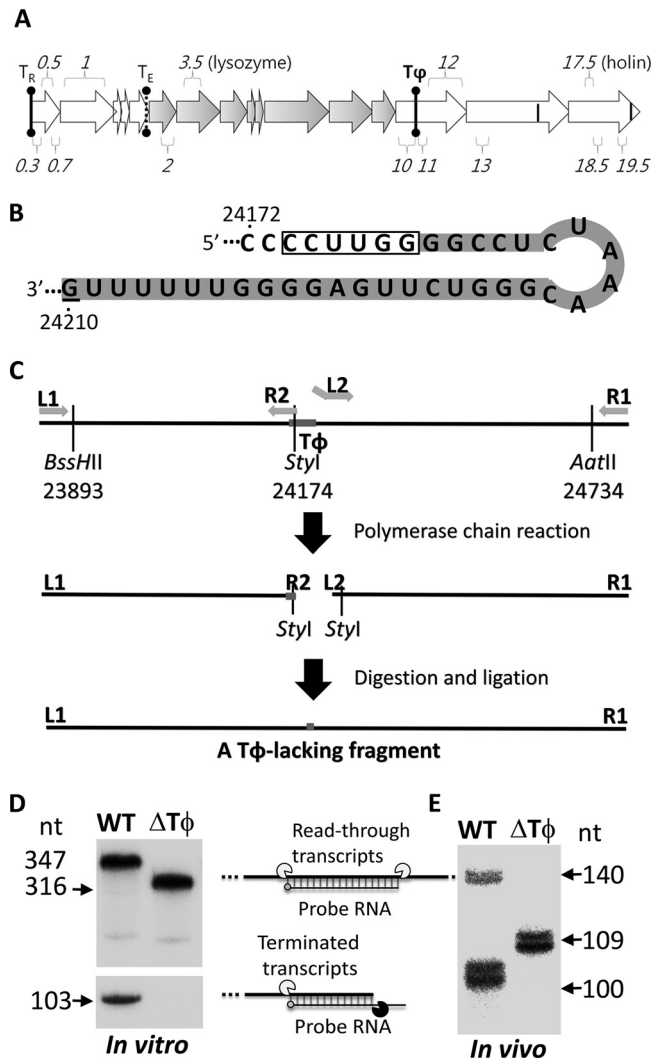


FIG 1 Terminator T ϕ -lacking mutant of T7 phage. (A) Major transcripts from phage T7 genome. Arrows represent RNase III-processed transcripts of T7 class I (empty arrows on the left), II (gray arrows in the middle), and III (empty arrows on the right) genes, and each arrow starts at a promoter and ends at an RNase III cleavage site. Where multiple promoters are present between two RNase III cleavage sites, extra vertical bars are shown within arrows. Two terminators for T7 RNA polymerase (T_R at the beginning of the genome and T ϕ in the middle of the class III region) are shown with a solid-line dumbbell, while *E. coli* RNA polymerase terminator T_E is shown with a dotted-line dumbbell. Among the 56 known or potential genes, only several genes mentioned in the text are shown. (B) RNA hairpin of the intrinsic terminator T ϕ . Transcription terminates with the G residue at genomic position 24210. The shaded 31 residues (from positions 24180 to 24210) are deleted in the T ϕ -lacking mutant. The StyI recognition sequence is boxed. (C) Strategy employed to create a T ϕ -lacking fragment used in constructing a T ϕ -lacking T7 mutant phage. (D) Confirmation of T ϕ deletion using *in vitro* multiple-round transcription reactions. (E) Confirmation of T ϕ deletion in *E. coli* after infection using RNase protection assays.

manner, first by *E. coli* RNA polymerase and then by T7 RNA polymerase.

The lysis machinery of phage T7 comprises three components: holin (gp17.5), lysozyme (gp3.5), and possible spanins (gp18.5 and gp18.7). While deletion of the lysozyme greatly delayed lysis, there was no significant change in lysis time in the absence of holin (10). Spanins are needed for lysis only at high salt concentrations

(11). During infection, the phage gene expression timings and levels are tightly regulated, mainly at transcriptional levels, by coupling of transcription with translocation of phage DNA. This suggests the importance of terminator T ϕ in maintaining the balance of expression among T7 genes during phage infection.

In this study, we show that the presence of terminator T ϕ is required to maintain normal burst size and lysis time of phage T7 during infection of *E. coli*. We observed that deletion of terminator T ϕ from T7 phage led to a reduction in phage burst size and a delay in lysis time, which could not be explained by any known lysis pathway of T7 phage. Instead, the T ϕ -lacking mutant was able to recover the wild-type lysis phenotype by deleting an early gene cluster. The results suggest for the first time the physiological roles of terminator T ϕ during phage infection.

MATERIALS AND METHODS

Bacteria and phage preparation. *E. coli* BL21 (Stratagene) was used as the host organism for T7 phage preparation and infection. T7 phage was purchased from the ATCC (BAA-1025-B2). A T ϕ -lacking T7 phage mutant was constructed with a deletion of 31 bp of the T ϕ terminator. Revertants 1 through 5 were evolved from the T ϕ -lacking mutant phage. The Δ 0.5-0.7 mutant of the T7 phage was constructed from revertant 1 by inserting the wild-type T ϕ terminator sequence back into its genome. Phage lysate was prepared by adding either one drop of archival phage stock or a single plaque into a BL21 host culture with an absorbance at 595 nm (*A*₅₉₅) of ~0.5 and shaking until the culture was clarified. NaCl was added to a final concentration of 1 M, and the total lysate was centrifuged. The supernatant was then passed through a 0.45- μ m syringe-driven filter unit (Millipore). Phage titer was measured using a soft-agar assay as previously described (12). *E. coli* DH5 α (Invitrogen) was used to make T ϕ -lacking phage mutants and for DNA cloning.

Assembly of T7 mutant genomes. For the T ϕ -lacking T7 mutant, a mutant phage of T7 lacking a 31-bp segment of T ϕ was constructed and assembled (Fig. 1C) using a previously described strategy (13). In brief, a T ϕ upstream part (amplicon L1-R2 from genomic positions 23823 to 24176) and a T ϕ downstream part (amplicon L2-R1 from genomic positions 24211 to 24801) were juxtaposed using StyI digestion and ligation. The resulting T ϕ -lacking fragment of 979 bp was then ligated into the T7 genome using two single-cut sites, BssHIII (cut at genomic position 23893) and AatII (cut at 24734), to make a T ϕ -lacking genome. The 40-kb T ϕ -lacking T7 genomic DNA was then transfected into competent DH5 α cells and plated on soft agar to allow plaque formation. Plaque PCR was then performed, and the PCR products were purified and sequenced to screen the correct mutant. The correct plaque was then used to make primary phage lysate. For making Δ 0.5-0.7 T7 phage from revertant 1, the wild-type sequence of the T ϕ terminator was inserted back in the genome using the same AatII and BssHIII sites.

Measurement of transcription termination efficiency. *In vitro* transcription termination efficiency was measured on DNA templates amplified from plasmid pKM01 (3) having either the wild-type terminator T ϕ or a T ϕ -lacking fragment cloned into the KpnI/HindIII site. The transcription reaction was carried out in a 20- μ l reaction mixture containing 0.5 μ g template DNA, 40 mM Tris-HCl, pH 7.9, 6 mM MgCl₂, 100 mM KCl, 10 mM dithiothreitol, 0.5 mM ribonucleotide triphosphates, 20 μ Ci [α -³²P]UTP (PerkinElmer), 4 U of RNasin (Promega), and 40 U of phage T7 RNA polymerase. The reaction mixture was incubated at room temperature for 1 h before being stopped by adding 10 μ l of loading buffer (10 mM EDTA, 12 M urea, 0.025% bromophenol blue, and 0.025% xylene cyanol). To measure *in vivo* transcription termination efficiencies, an RNase protection assay was performed as previously described (3). Products were analyzed on 8 M urea-12% polyacrylamide gels. Band intensities were analyzed using TINA 2.0 software (DesignSoft).

Measurement of burst size. Phage burst size was measured as previously described (14), with some modifications. Briefly, a BL21 culture at

an A_{595} of 0.3 to 0.4 was infected with phages at a multiplicity of infection (MOI) of 0.02. After allowing phages to be attached to host cells for 2 min, the culture was centrifuged to discard all free phages and quickly diluted 10,000-fold into prewarmed media. Titers of samples at 6 and 8 min after infection were determined to obtain the number of infective centers. The culture was further diluted 100-fold, samples were extracted every 2 min from 15 to 25 min after infection and treated with chloroform, and the titers were determined. The burst size of individual cells was calculated by fitting the data from three independent experiments into a sigmoidal curve model using the least-squares fitting method.

Kinetic assay of lysis. An *E. coli* BL21 culture at an A_{595} of 0.2 to 0.3 was infected with phages at an MOI of 3 to 4. Adsorption was allowed to proceed for 2 min at 37°C, and the culture was centrifuged to eliminate all free phages. The pellet was resuspended in a new medium and allowed to grow at 37°C in a shaking incubator. At every 2 min after infection, 200 μ l of the culture was removed and placed in a 96-well plate kept on ice. Cell density was measured by A_{595} after all samples were taken. Lysis curve and average lysis time were estimated by fitting the data from at least two independent experiments into a sigmoidal curve using the least-squares fitting method.

Measurement of adsorption rates. Freshly prepared phages were added to the BL21 culture at an A_{595} of \sim 0.3 at an MOI of 0.001. After 5 min of infection at 37°C, 200 μ l of culture was taken and quickly centrifuged to separate free phages from adsorbed phages. Another 200 μ l of culture was taken at the same time and kept unspun. Both spun and unspun suspensions were then separately added to 2.5 ml of 0.7% LB agar and plated onto 1.5% LB agar to determine the total number of phages (N_t) from the unspun sample and the number of free phages (N_f) from the spun sample. The adsorption rate, α , was calculated using the equation $\alpha = -0.2[\ln(N_f/N_t)]$.

Reverse Northern blot. Total RNA was extracted from cell lysate samples using a RiboPure-bacteria kit (Ambion). Phage mRNA levels were measured semiquantitatively by reverse Northern blotting as previously described (15), except for the blotting step. For blotting, DNA probes first were amplified using PCR. The products were then denatured by boiling for 5 min in the presence of 0.35 M sodium hydroxide before being neutralized with 0.5 M sodium acetate. The denatured probe (100 μ l) was then dot blotted onto a Zeta-Probe blotting membrane (Bio-Rad) by following the manufacturer's instructions. Radioactive intensity was measured and analyzed using TINA 2.0 software (DesignSoft).

Quantification of phage mRNA levels. The postinfection cellular levels of phage gene mRNAs were measured quantitatively by reverse transcription followed by quantitative real-time PCR (RT-qPCR). The first-strand cDNA was synthesized from 1 μ g of total RNA using biotinylated random 15-mer primers and reverse transcriptase ImpromII (Promega) and purified using streptavidin-coated magnetic beads (Dynabeads M-280; Invitrogen). Quantitative PCR for targeted mRNAs was performed using a CFX Connect system (Bio-Rad). All statistical analyses were performed using two-tailed Student's *t* tests.

Overexpression of holin gene. A 204-bp segment of T7 gene 17.5 (encoding holin) was amplified from T7 genome DNA using a forward primer (5'-ATCACATATGCTATCATTAGACTTTAAACAA-3') and a reverse primer (5'-ATCACTCGAGTCACTCCTTATTGGCTTCTT-3') in PCR consisting of 35 cycles of denaturation at 95°C, annealing at 57°C, and extension at 72°C, each for 30 s. The resulting amplicon was cloned into the NdeI/XhoI site of the pET21 vector, and BL21 was transformed with the resulting plasmid for overproduction of T7 holin during phage infection.

Passaging of phage for experimental evolution. Passaging was done by adding phage to 4 ml of log-phase BL21 host (A_{595} of 0.2 to 0.5) at low MOI ($<$ 0.1). The culture was then shaken at 37°C for 30 to 60 min. A drop from the previous passage was then added to 4 ml of a new log-phase host culture, and the process was repeated. If a phage stock was kept overnight before the next passage, 10 μ l of chloroform was added and the final lysate was kept at 4°C.

RESULTS

A T7 phage mutant lacking terminator T ϕ . We created a T7 phage mutant lacking the transcription terminator T ϕ to understand the roles of T ϕ in the phage T7 lytic cycle. As T ϕ is a typical class I intrinsic terminator (4), both the hairpin structure and immediate downstream U stretch are required for functionality. We deleted a 31-bp DNA sequence (from genomic positions 24180 to 24210) encoding the terminator hairpin (including five residues in the upstream side of the stem, all six in the loop, and all 13 in the downstream side of the stem), the following six U residues, and the termination site G residue of T ϕ from T7 genome (Fig. 1B). A T ϕ -lacking fragment was constructed by combining a T ϕ upstream part and a T ϕ downstream part through an StyI site (Fig. 1C). The combined fragment was inserted into the unique BssHIII and AatII sites of the T7 genome before being transfected into competent *E. coli* DH5 α for assembly of the T ϕ -lacking mutant phage.

The deletion of T ϕ was confirmed by DNA sequencing and *in vitro* and *in vivo* transcription assays (Fig. 1D). For *in vitro* multiple-round transcription assays, a 238-bp T ϕ -containing segment from genomic positions 24153 through 24390 or a corresponding 207-bp T ϕ -lacking segment was cloned from the wild-type or mutant phage, respectively, into the KpnI/HindIII site of a ϕ 10 promoter-containing plasmid, pKM01, so that 347- or 316-nucleotide (nt)-long runoff transcripts, respectively, could be produced by T7 RNA polymerase transcription using PCR amplicon templates. The wild-type template yielded both 103-nt terminated transcripts and 347-nt runoff transcripts, showing a termination efficiency of 77% (Fig. 1D). In contrast, transcription of the T ϕ -lacking template did not produce any terminated transcripts other than 316-nt runoff transcripts.

This *in vitro* confirmation agreed with measurements of *in vivo* transcripts using an RNase protection assay (Fig. 1E). For preparation of RNA probes, a T ϕ -containing template (genomic positions from 24111 to 24250) and a corresponding T ϕ -lacking template were amplified using PCR of the wild-type and mutant phages, respectively. The reverse primer had a ϕ 10 promoter in the 5' overhang for phage T7 RNA polymerase transcription of the amplicons to produce radioactively labeled 140- and 109-nt-long RNA probes, respectively, complementary to the T7 transcripts produced after infection.

The total RNA transcripts were extracted from *E. coli* BL21 infected with the wild-type or mutant phage and mixed with respective radiolabeled probes before being treated with a mixture of single-strand-specific RNases A and T1. The 140-nt RNA probe was hybridized to both terminated and read-through transcripts to yield protection of 100 and 140 nt, respectively, among total transcripts from the wild-type phage. In contrast, only the read-through transcripts were detected (109 nt protection) among those from the mutant phage. Accordingly, the mutant phage was demonstrated to lack the terminator T ϕ physically and functionally.

Burst shrinkage and lysis delay by T ϕ deletion. With respect to the life of a lytic phage, two important indexes are burst size and lysis timing. Burst size is the number of progeny particles a phage produces after one cycle of infection, and lysis timing shows how fast or slow a phage lyses its bacterial hosts, in other words, how short or long the latent period is. These two parameters are normally regulated in a trade-off manner to maximize phage fitness:

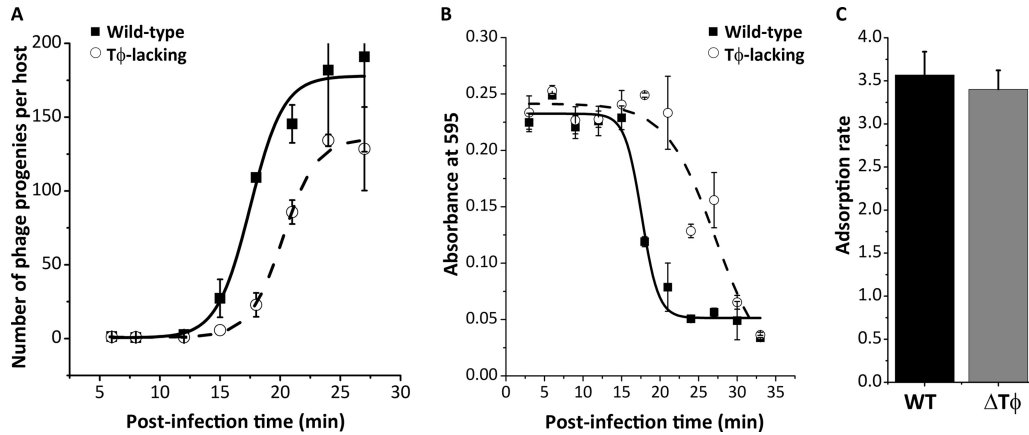


FIG 2 Phenotypic characterization of the T ϕ -lacking mutant phage. (A) One-step growth curve for burst size measurement of the wild-type (filled squares, solid line) and T ϕ -lacking (open circles, dash line) phages. (B) Lysis curves for estimation of lysis time from the wild-type and T ϕ -lacking mutant phage infections. (C) Adsorption rates measured for the wild-type (black column) and T ϕ -lacking (gray column) phages. Vertical error bars represent standard deviations.

small bursts are compensated for by fast lysis, while delayed lysis usually yields enlarged bursts (16–20).

When measured under conditions where the host *E. coli* BL21 was in an early log phase, the burst sizes of wild-type and T ϕ -lacking mutant phages were 179 and 130 particles per host, respectively (Fig. 2A and Table 1). The T ϕ -lacking mutant achieved only 73% of the burst size of the wild-type phage ($P = 0.01$). The burst shrinkage was, however, accompanied by a significant delay rather than acceleration of lysis of the T ϕ -lacking mutant ($P = 0.0003$). Lysis was delayed by approximately 10 min in the mutant compared to the wild-type phage, with the calculated mean lysis time shifted from 16.8 to 26.9 min, when BL21 was infected at 37°C (Fig. 2B and Table 1). Thus, deletion of T ϕ from the T7 genome led to a delay in lysis time but shrinkage in burst size, both of which are disadvantageous for the phage adaptation to environments.

Phage adsorption rate unchanged by T ϕ deletion. Optimal lysis time is positively correlated with adsorption rate, and phage strains with higher adsorption rates show faster lysis than those with lower adsorption rates (21). To determine if the adsorption rate provides any contribution to the delay in lysis time observed with the T ϕ -lacking mutant, we measured adsorption rates of the wild-type and mutant phages in infection of exponentially growing BL21 at 37°C (Fig. 2C and Table 1). No significant difference in adsorption rate was observed ($P = 0.45$) between the wild type ($3.6 \text{ phage}^{-1} \text{ ml}^{-1} \text{ min}^{-1}$) and T ϕ -lacking mutant ($3.4 \text{ phage}^{-1} \text{ ml}^{-1} \text{ min}^{-1}$). These results are consistent with a previous finding that adsorption of T7 requires only the phage tail fiber protein (gp17) interacting with *E. coli* surface lipopolysaccharide (1), be-

cause deletion of the terminator T ϕ would not change the tail protein.

Global elevation of T ϕ downstream gene expression by T ϕ deletion. Because the terminator T ϕ is located just upstream of gene 11 in the middle of the T7 genome (Fig. 1A), deletion of T ϕ was expected to lead to elevated expression of T ϕ downstream genes starting from gene 11, in the middle of the genome, through gene 19.5, at the end of the genome. To test this hypothesis, reverse Northern blotting was performed using 15 DNA probes of 200 to 500 bp designed specifically for T7 genes of classes I (early), II (middle), and III (late) to semiquantitatively measure their mRNA levels in the total RNA extracts from *E. coli* BL21 at 10 min after infection with the wild-type or mutant phage (Fig. 3A).

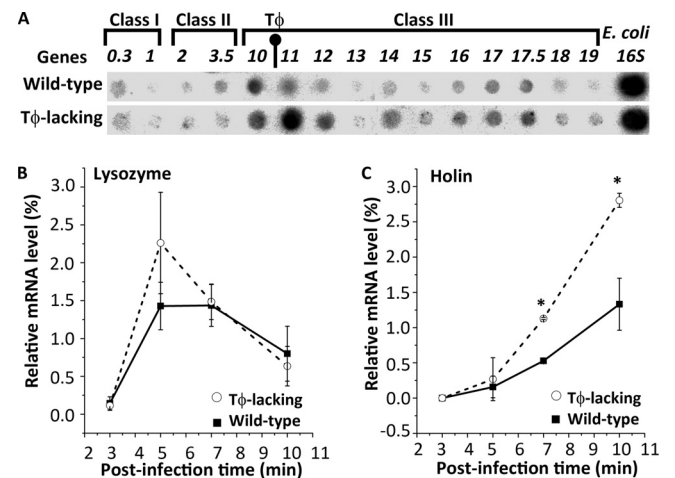


FIG 3 Transcription profiling of T7 genes during infection of the wild-type and T ϕ -lacking phage. (A) The mRNA levels of several T7 genes in the wild-type and T ϕ -lacking phage infections were measured using reverse Northern blotting. *E. coli* 16S rRNA was used as a normalization control. The layout of spots follows the sequence of T7 genes in the genome shown at the top, and terminator T ϕ is located between genes 10 and 11. (B and C) The mRNA levels of two T7 lysis proteins, lysozyme (B) and holin (C), in the wild-type (filled squares) and T ϕ -lacking phage infections (empty circles) were quantified in a time course manner using RT-qPCR.

TABLE 1 Phenotypic characterization of wild-type and mutant T7 phages^a

Phage T7 type	Burst size (phage cell ⁻¹)	Lysis time (min)	Absorption rate (phage ⁻¹ ml ⁻¹ min ⁻¹)
Wild type	179 ± 19 (reference)	16.8 ± 0.7 (reference)	3.6 ± 0.3 (reference)
T ϕ -lacking	130 ± 06 ($P = 0.01$)	26.9 ± 0.3 ($P = 0.0003$)	3.4 ± 0.2 ($P = 0.5$)
Revertant 1	170 ± 14 ($P = 0.4$)	13.9 ± 0.6 ($P = 0.004$)	Not determined
$\Delta 0.5-0.7$	184 ± 13 ($P = 0.2$)	12.6 ± 0.2 ($P = 0.003$)	Not determined

^a P values were calculated against the wild type.

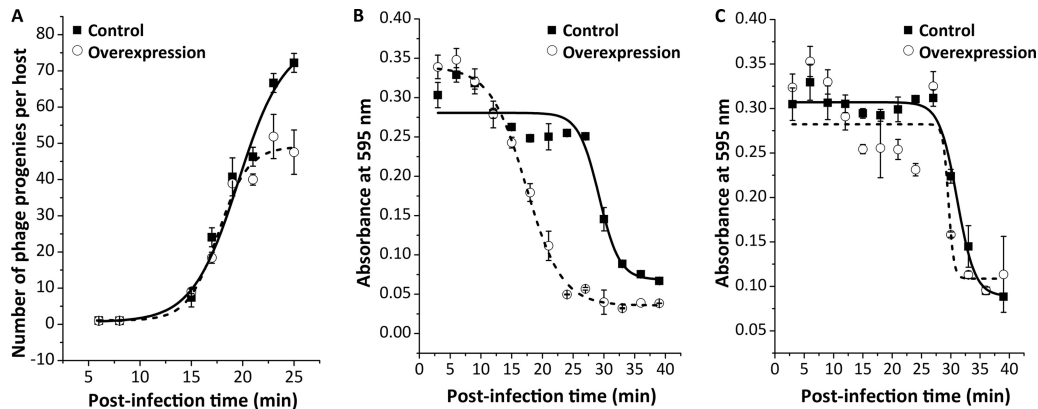


FIG 4 Effects of holin overproduction on burst size and lysis time of T7 phage infection. (A and B) The T7 phage burst size (A) and lysis time (B) were measured with infection of the BL21 host carrying a pET21 vector containing the holin gene under 1.5 mM IPTG-induced (empty circles, dash line) and noninduced (filled squares, solid line) conditions. (C) A control experiment measuring T7 lysis time with infection of the BL21 host carrying an empty pET21 vector under the same conditions.

Ten of the tested T ϕ downstream genes, 11, 12, 13, 14, 15, 16, 17, 17.5, 18, and 19, were upregulated in the T ϕ -lacking mutant infection compared to the wild-type phage infection. The extent of upregulation ranged from 1.3- to 6.8-fold after normalization against the levels of *E. coli* 16S rRNA, an internal control. Maximum elevation was observed for genes 11 and 12; this finding is conceivable because these two promoter-lacking genes rely entirely on read-through at T ϕ for their expression, whereas the other genes can be transcribed from their own promoters in addition to the read-through at T ϕ .

In contrast, four genes of the early and middle classes located upstream of T ϕ in the T7 genome showed either no expression change (genes 0.3 and 3.5) or mild upregulation (genes 1 and 2) in the T ϕ -lacking mutant infection. Expression of gene 10, located just upstream of T ϕ , also appeared unchanged in the mutant phage infection. Thus, many T7 genes were upregulated to various degrees, but most T ϕ downstream genes, especially the T ϕ -dependent genes 11 and 12, were highly upregulated in the T ϕ -lacking mutant phage infection compared to the wild-type phage infection.

mRNA levels of T7 lysis proteins, lysozyme, and holin. Lysis is the destruction of the host cell wall and membrane. It releases phage progenies; therefore, it is essential for phage propagation. The lysis machinery in most phages with large double-stranded DNA consists of two components under normal salt concentrations: a lysin, a muralytic enzyme that degrades the cell wall, and a holin, a transmembrane protein that creates lesions in the host inner membrane through which lysin can gain access to the cell wall (22). In phage T7, these proteins are lysozyme encoded by a middle gene, 3.5, and holin encoded by a late gene, 17.5.

The mRNA levels of these two lysis proteins were quantitatively measured in a time course manner after infection started using RT-qPCR (Fig. 3B). The lysozyme mRNA level was not significantly different between the wild-type and T ϕ -lacking mutant phages throughout the infection course ($P > 0.05$ at all time points tested). Accordingly, lysozyme levels are not associated with delayed lysis or downsized burst observed in the T ϕ -lacking mutant infection.

On the other hand, the holin mRNA level was significantly elevated in the T ϕ -lacking mutant infection, reaching a 2.1-fold

increase at 7 ($P = 0.002$) and 10 min ($P = 0.003$) after infection (Fig. 3C). To assess if the holin upregulation was the cause of lysis delay and burst downsizing in the T ϕ -lacking mutant infection, we overexpressed the holin gene in the *E. coli* BL21 host during the wild-type phage infection and measured lysis time and burst size.

Burst shrinkage but lysis acceleration by holin overproduction. BL21 was transformed to take up a pET21 plasmid carrying the holin gene and infected with the wild-type phage, while holin gene overexpression was induced by adding 1.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) or noninduced as a control. The burst size was 50 versus 80 progenies/host under induced and noninduced conditions, respectively (Fig. 4A); therefore, it was significantly reduced (by 37%) by overproduction of holin ($P = 0.02$). Accordingly, the burst shrinkage observed in the T ϕ -lacking mutant infection could result from upregulation of holin gene expression, a consequence of T ϕ deletion.

However, the holin overproduction significantly accelerated rather than delayed lysis of the wild-type phage ($P = 0.003$), as the calculated mean lysis time was 16.5 and 28.2 min under the induced and noninduced conditions, respectively (Fig. 4B). The lysis acceleration was a bona fide effect of holin overproduction, because there was no difference in lysis time when BL21 was transformed with an empty pET21 vector: 25.7 and 27.0 min under induced and noninduced conditions, respectively (Fig. 4C). Accordingly, the lysis delay observed in the T ϕ -lacking mutant infection cannot result from the upregulation of holin gene expression, which leads to an opposite consequence in the wild-type phage infection.

Furthermore, all of these results together suggest that overexpression of multiple T ϕ downstream genes, including the holin gene, had a greater effect than merely counteracting the lysis-accelerating effect of holin gene overexpression to result in a further delayed lysis. Accordingly, a yet-unknown factor(s) other than lysozyme and holin is involved in causing the lysis delay in the T ϕ -lacking mutant infection.

Lysis time and burst size recovered by deletion of T7 early genes. Because the known lysis pathway of T7 phage involving lysozyme and holin could not explain the lysis delay, we took the classical genetics approach of experimental evolution. The T ϕ -lacking mutant phage with a delayed lysis property was passed

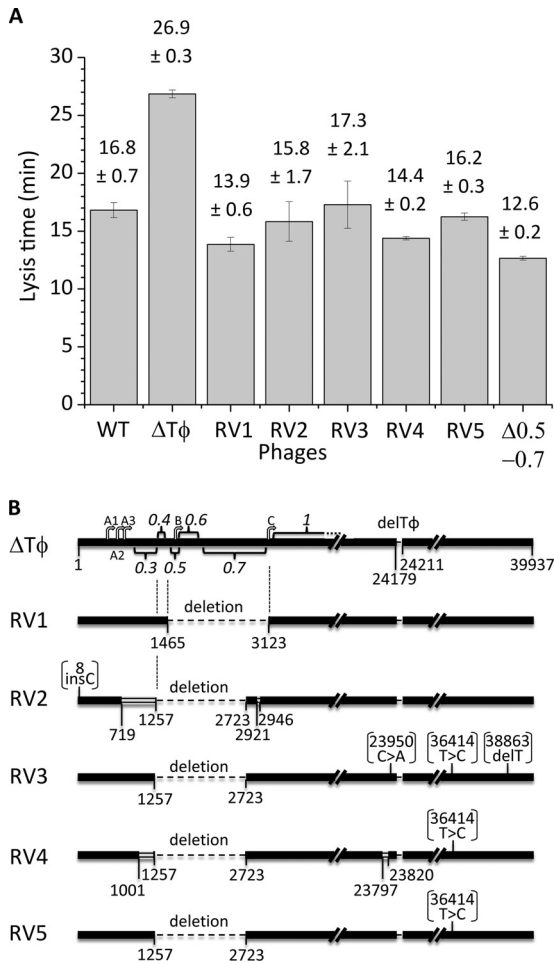


FIG 5 Experimental adaptation of the Tφ-lacking mutant. (A) Lysis time was measured for the wild-type (WT) and Tφ-lacking (ΔTφ) phages, the five fast-lysing revertants (RV1 through RV5), and the early-gene-lacking control (Δ0.5-0.7). (B) Genome maps of the ancestral Tφ-lacking mutant (ΔTφ) and the five revertants (RV1 through RV5). Relative genomic positions of some *E. coli* promoters (A1, A2, A3, B, and C) and T7 genes (0.3, 0.4, 0.5, 0.6, 0.7, and 1) are shown for ΔTφ on the top. Dashed lines indicate deleted regions. Double bars indicate heterologous regions. Point mutations shown in parentheses are either synonymous or intergenic.

consecutively through a series of log-phase hosts until some spontaneous mutations rendered lysis as fast as that of the wild-type phage. Five independent adaptations were performed to reveal common suppressor mutations.

In the first adaptation, a fast-lysing revertant arose at passage 29, here referred to as revertant 1. The other four revertants, revertants 2 to 5, arose at passage 27 of their respective adaptations. All five revertants lysed their hosts significantly faster than the ancestral Tφ-lacking mutant with a lysis time of 26.9 min ($P < 0.05$) (Fig. 5A). Revertants 1 (13.9 min) and 4 (14.4 min) lysed even faster than the wild-type phage, with a lysis time of 16.8 min ($P = 0.004$ and 0.016 , respectively), whereas revertants 2 (15.8 min), 3 (17.3 min), and 5 (16.2 min) lysed as fast as the wild type ($P > 0.05$).

To identify the mutations that recovered fast lysis, we sequenced the entire genomes of all five revertants (Fig. 5B). Revertant 1 carried a deletion from genomic positions 1466 to 3123 (harboring genes 0.5, 0.6, and 0.7). Revertants 2, 3, 4, and 5 commonly carried a larger deletion from positions 1255 to 2722 (genes 0.3 through 0.7) and a synonymous substitution at position 36414 (T to C within gene 17.5). Additionally, revertant 2 carried an addition of C at position 8 and revertant 3 carried a substitution at 23950 (C to A), an addition of G at 35299, and a deletion of T at 38863. Revertant 4 additionally carried a substitution at 297 (T to A), a deletion of G at 23800, an addition of A at 23818, and a substitution at 23820 (G to T), but revertant 5 carried no additional changes.

Thus, the mutations commonly shared by all five revertants were deletion of three nonessential genes, 0.5, 0.6, and 0.7, spanning 1,257 bp from positions 1466 to 2722, suggesting that deletion of these three early genes was sufficient to suppress the lysis delay effect of Tφ deletion. Because the deletion in revertant 1 affected only three genes while the other revertants lacked more genes, we further characterized revertant 1 as a representative revertant. Revertant 1 also recovered the wild-type burst size, reaching 170 progenies/host ($P = 0.4$) (Fig. 6A and Table 1), substantially larger than the Tφ-lacking mutant (130 phages/host). These results together showed that deletion of three nonessential early genes, 0.5, 0.6, and 0.7, could effectively suppress both lysis delay and burst-downsizing effects of Tφ deletion.

To assess the effect of deletion of these early genes, 0.5 to 0.7,

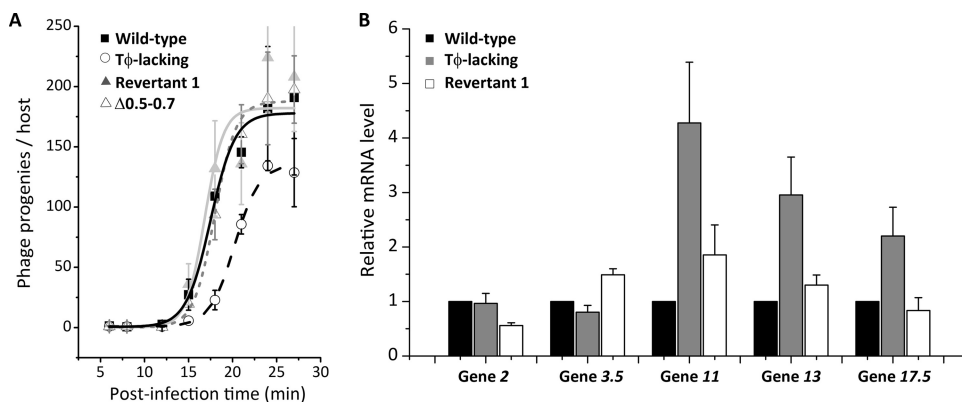


FIG 6 Characterization of revertant 1. (A) Burst size measurement in wild-type T7 (filled square), Tφ-lacking mutant (empty circle), revertant 1 (filled triangle), and Δ0.5-0.7 (empty triangle). (B) The mRNA levels of five T7 genes, 2, 3.5, 11, 13, and 17.5, were quantified at 10 min after infection in wild-type T7 (black), Tφ-lacking mutant (gray), and revertant 1 (white) using RT-qPCR.

alone in the presence rather than the absence of T ϕ terminator, we inserted the wild-type T ϕ sequence back into revertant 1, resulting in a mutant designated $\Delta 0.5-0.7$. The burst size of this control mutant was 184 phage progenies/host (Table 1), not significantly different from that of the wild type ($P = 0.2$). In contrast, the lysis time was 12.6 min (Table 1), significantly faster than that of the wild type ($P = 0.003$). Thus, the effect of deletion of genes *0.5* to *0.7* alone was to accelerate lysis while hardly affecting burst size.

Burst size associated with holin gene expression level. Deletion of T ϕ led to upregulation of the holin gene *17.5* (Fig. 3B), which in turn led to premature breakage of *E. coli* host and reduced burst size. Since revertant 1 recovered the burst size, we examined whether the holin expression level was downregulated in revertant 1 infection compared to the T ϕ -lacking mutant infection. We measured mRNA levels of holin at 10 min after infections using RT-qPCR (Fig. 6B). The holin mRNA level was increased 2-fold in the T ϕ -lacking mutant infection compared to the wild-type phage infection but decreased in revertant 1 infection to a level comparable to that of the wild-type phage infection. Consistent with a finding that the burst size was associated with the holin expression level (Fig. 3B), these results could explain how the burst size was recovered in revertant 1.

Lysis time associated with T ϕ downstream gene expression levels. Although lysis time was not associated with the holin gene expression level (Fig. 4B), most other T ϕ downstream genes were upregulated by T ϕ deletion (Fig. 3A). Thus, mRNA levels of two other T ϕ downstream genes, *11* and *13*, were quantitatively measured (Fig. 6B). Their mRNA levels were increased in the T ϕ -lacking mutant infection compared to the wild-type phage infection but decreased again in revertant 1 infection compared to the T ϕ -lacking mutant infection. In contrast, mRNA levels of two T ϕ upstream genes, *2* and *3.5* (as controls), were similar in the wild-type, T ϕ -lacking mutant and revertant 1 infections. These results suggest that lysis time is associated with mRNA levels of T ϕ downstream genes (other than the holin gene) rather than T ϕ upstream genes (including the lysozyme gene).

DISCUSSION

This study is the first to address the physiological role of the phage T7 terminator T ϕ during infection of *E. coli*. We report that direct effects of T ϕ deletion from the phage T7 genome are a delay in lysis time accompanied by a shrinkage rather than an enlargement of burst size at the phenotypic level (Fig. 2) and global elevation in mRNA levels of most, if not all, T ϕ downstream genes at the molecular level (Fig. 3). All of these molecular and phenotypic effects of T ϕ deletion could be suppressed by deletion of early genes, especially genes *0.5*, *0.6*, and *0.7* (Fig. 5 and 6). Burst size appeared to be associated with cellular levels of T7 holin, a T ϕ downstream gene product, but lysis time was associated with T ϕ downstream gene product(s) other than holin; this suggests that phage T7 lysis involves an unknown factor(s) other than the known lysis proteins of phage T7.

Holin creates sudden membrane lesions in a concentration-dependent manner on the phage lysis pathway (20, 23–25). This saltatory manner of holin action is important to ensure that production of phage progenies unabatedly continues until the moment of lysis. A straightforward interpretation of the observed shrinkage of burst in the T ϕ -lacking mutant infection (Fig. 2A) and recovery of burst size in the revertant 1 infection (Fig. 6A) would be that T ϕ deletion results in fast accumulation of holin,

which directly leads to two consequences: first, early saltatory inner cell membrane leakage that allows lysozyme to pass through and degrade the cell wall, causing fast lysis; and second, premature cessation of progeny production, leading to burst downsizing. We observed both phenomena when holin-overproducing *E. coli* cells were infected with the wild-type T7 phage (Fig. 4A), providing further evidence supporting holin association with burst size. This is the first report that the presence of the phage T7 terminator T ϕ is required for maintaining optimal burst size during T7 infection of *E. coli*.

There are many factors, both external and internal, that regulate phage burst size in its infection cycle, as the lysis function is strictly regulated in phages (26). Internally, burst size (b) is determined by the length of the eclipse period (e), the rate of maturation (m), and lysis time (l), approximately following the equation $b = m(l - e)$ (19). The correlation between burst size and lysis time would not always be this linear, however, because burst size would reach saturation if the phage has exhausted all of the resources in the host cell. In general, nevertheless, burst size increases when lysis time increases, as lysis is delayed for larger bursts in order to maintain its maximal adaptiveness. Thus, burst size and lysis time are in a simple trade-off relation. In fact, holin overproduction, which led to downsized burst, resulted in accelerated lysis in the wild-type phage infection (Fig. 4B).

Surprisingly, however, in the T ϕ -lacking mutant infection, the burst was smaller even though lysis was substantially delayed compared to the wild-type phage infection (Fig. 2). Thus, with T ϕ deletion both burst size and lysis time are far from optimal values, and the trade-off relationship between the two parameters no longer holds. Lysis requires degradation of not only the inner cell membrane by holin but also the cell wall and the outer cell membrane by lysozyme (and spanins, under certain conditions). The rate-limiting step in the retarded lysis of the T ϕ -lacking mutant lies in either the cell wall or the outer membrane degradation step, although the mechanism remains elusive.

It is known that release of T7 lysozyme is spontaneous upon holin-induced membrane lesion. T7 lysozyme is known to interact only with T7 RNA polymerase and with itself (27), which is important for its transcription-inhibitory function but not for its degradation of the host cell wall. Lysis was not delayed but rather accelerated by holin overproduction in the wild-type phage infection (Fig. 4B), suggesting that overexpression of multiple T ϕ downstream genes did more than counteract the effect of holin overexpression on lysis time. Accordingly, it is suggested that some unknown factors other than holin, lysozyme, and spanins are involved in the lysis pathway of T7 phage. Heineman et al. (14) also suggested that T7 contains additional holins, as their optimality failed to predict the lysis time of T7 phage.

Previous studies have shown the power of fast evolution in a given phage group to overcome disadvantages caused by mutation or deletion of some beneficial gene functions (28, 29). Similarly, in our search for unknown factors that can overcome delay in lysis and shrinkage in burst size caused by T ϕ deletion, suppressor mutations of T ϕ deletion obtained through experimental evolutions were identified in this study (Fig. 5 and 6). According to the optimality model (30), a given trait can reach its optimal value through simple natural selection when placed under a few selective forces. The slow-lysing T ϕ -lacking mutant phage successfully adapted fast lysis in all five independent evolution experiments.

Deletion of three early nonessential genes, *0.5*, *0.6*, and *0.7*,

from the T ϕ -lacking genome was sufficient to suppress both of the two disadvantageous phenotypes, namely, delayed lysis and downsized burst of T ϕ -lacking mutant phage, suggesting that one or more of these gene products can reverse the action. The deletion of this early gene cluster could have two effects on T7 infection. First, the T7 genome is shortened to reduce the time taken for genome penetration into host cells, which would accelerate lysis (31). Second, the three gene products, gp0.5, gp0.6, and gp0.7, are not produced; therefore, their function(s) and effect(s) on infection would be absent. Whereas the functions of gp0.5 and gp0.6 are not known yet, gp0.7 is known to have a protein kinase activity that can ultimately affect the expression of phage genes.

A serine-threonine protein kinase encoded by the T7 gene *0.7* phosphorylates many host proteins of the transcription (such as *E. coli* RNA polymerase β and β' subunits), RNA processing (such as RNases III and E), and translation machineries (such as elongation factors G and P and ribosomal proteins S1 and S6). This phosphorylation was reported to help inactivate host *E. coli* transcription and stabilize T7 mRNAs for increased translation (32, 33). If so, deletion of gene *0.7* should reduce T7 mRNA levels, among other effects. In fact, deletion of gene *0.7* and others from the T ϕ -lacking mutant genome reduced the holin mRNA level in revertant 1 infection to a level comparable to that of wild-type phage infection (Fig. 6B). In other words, burst downsizing is caused by deletion of T ϕ , leading to elevation of holin mRNA level (in the T ϕ -lacking mutant infection), and reversed by deletion of gene *0.7*, leading to reduction of the holin mRNA level (in revertant 1 infection). Accordingly, these data support that the burst size is associated with holin levels.

In contrast, lysis time appears to be associated with a yet-unknown T7 factor. The factor would be a T ϕ downstream gene product(s), because lysis was delayed when deletion of T ϕ elevated the mRNA levels of T ϕ downstream genes (Fig. 3A) but recovered when further deletion of genes *0.5* to *0.7* reduced mRNA levels of genes *11* and *13* (Fig. 6B) and presumably other T ϕ downstream genes as well. However, holin, a product of a T ϕ downstream gene (*17.5*), is not associated with lysis time, because the effect of holin overproduction was opposite that in the wild-type phage infection (accelerating lysis) (Fig. 4B) and the T ϕ -lacking mutant infection (delaying lysis) (Fig. 2B).

Among the 15 genes located downstream of T ϕ in the T7 genome, nine genes, *11*, *12*, *13*, *14*, *15*, *16*, *17*, *18*, and *19*, are essential, and their gene products all act on the assembly of progeny phages (1). Three other genes, *17.5*, *18.5*, and *18.7*, are regarded as non-essential, but their functions are known. Gene *17.5* encodes holin (of type II), which makes holes or channels in the inner membrane so that the lysozyme (gp3.5) can reach the cell wall and degrade it. The overlapping genes, *18.5* and *18.7*, apparently encode spanins, which span the periplasm and are involved in disrupting the outer cell membrane under high salt concentrations, and they are homologous to the overlapping genes Rz and Rz1 of phage λ (11). The remaining three genes, *19.2*, *19.3*, and *19.5*, are hypothetical at present but conserved among close relatives of phage T7. If these hypothetical genes are ever expressed as proteins or noncoding RNA, they can be candidates for the unknown lysis time-associated factor, among many others, although loss of gene *19.5* has previously caused a reduced burst (1).

In summary, we have observed for the first time the roles of terminator T ϕ in maintaining burst size and lysis time of phage T7 during infection of *E. coli*. Phage T7 burst size is associated with T7

holin, as the burst is downsized by elevation of holin, which can be observed when the phage lacks T ϕ . T7 lysis time is not associated with holin or lysozyme but with an unknown T ϕ downstream gene product(s), which can be upregulated by T ϕ deletion, leading to delayed but not accelerated lysis. It will be worthwhile to search for a new T7 lysis time-associated factor.

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