

Rapid Degradation of Host mRNAs by Stimulation of RNase E Activity by Srd of Bacteriophage T4

Dan Qi,* Abdulraheem M. Alawneh,* Tetsuro Yonesaki,* and Yuichi Otsuka^{†,1}

*Department of Biological Sciences, Graduate School of Science, Osaka University, Toyonaka-shi, Osaka 560-0043, Japan, and

[†]Department of Microbiology, School of Medicine, Dokkyo Medical University, Mibu-machi, Shimotsuga-gun, Tochigi 321-0293, Japan

ABSTRACT *Escherichia coli* messenger RNAs (mRNAs) are rapidly degraded immediately after bacteriophage T4 infection, and the host RNase E contributes to this process. Here, we found that a previously uncharacterized factor of T4 phage, Srd (Similarity with *rpoD*), was involved in T4-induced host mRNA degradation. The rapid decay of *ompA* and *lpp* mRNAs was partially alleviated and a decay intermediate of *lpp* mRNA rapidly accumulated in cells infected with T4 phage lacking *srd*. Exogenous expression of Srd in uninfected cells significantly accelerated the decay of these mRNAs. In addition, *lpp*(T) RNA, with a sequence identical to the decay intermediate of *lpp* mRNA and a triphosphate at 5'-end, was also destabilized by Srd. The destabilization of these RNAs by Srd was not observed in RNase E-defective cells. The initial cleavage of a primary transcript by RNase E can be either direct or dependent on the 5'-end of transcript. In the latter case, host RppH is required to convert the triphosphate at 5'-end to a monophosphate. *lpp*(T) RNA, but not *lpp* and *ompA* mRNAs, required RppH for Srd-stimulated degradation, indicating that Srd stimulates both 5'-end-dependent and -independent cleavage activities of RNase E. Furthermore, pull-down and immunoprecipitation analyses strongly suggested that Srd physically associates with the N-terminal half of RNase E containing the catalytic moiety and the membrane target sequence. Finally, the growth of T4 phage was significantly decreased by the disruption of *srd*. These results strongly suggest that the stimulation of RNase E activity by T4 Srd is required for efficient phage growth.

KEYWORDS Srd; RNase E; *Escherichia coli*; bacteriophage T4; mRNA decay

BACTERIOPHAGE T4 shuts off gene expression of the host, *Escherichia coli*, immediately after infection and quickly starts to express its own genes (Kutter *et al.* 1994). Multiple mechanisms, such as modifications of apparatuses for transcription and translation, are involved in this shift of gene expression from *E. coli* to T4 (Carlson *et al.* 1994). Our previous work revealed that the representative stable *E. coli* messenger RNAs (mRNAs), *lpp* and *ompA*, were rapidly degraded after T4 infection (T4-induced host mRNA degradation) and that RNase E, which is an essential endoribonuclease in *E. coli* (Marcaida *et al.* 2006), primarily functions in T4-induced host mRNA degradation (Ueno and Yonesaki 2004). This rapid degradation of host mRNAs may contribute

to the quick shift from *E. coli* to T4 metabolism because it leads to immediate cessation of host gene expression and consequently generation of ribonucleotides and free ribosomes, each of which would stimulate transcription and translation of T4 genes. In fact, deficiency of RNase E resulted in a slow start of T4 gene transcription, reducing the level of transcription (Otsuka and Yonesaki 2005) and retarding the growth of T4 (Mudd *et al.* 1990a). In eukaryotic cells, the degradation of host mRNAs after viral infection, such as in alphaherpesvirus, gammaherpesvirus, or betacoronavirus, also contributes to the shutoff of host gene expression (Gaglia *et al.* 2012). In these cases, host mRNA degradation is initiated by a viral factor and requires host 5'–3' exoribonuclease Xrn1. Therefore, the degradation of host mRNAs after viral infection may be a common mechanism in both prokaryotes and eukaryotes; infection with virus activates the host mRNA degradation machinery for the shift of gene expression from host to virus.

Similar to eukaryotic viruses described above, T4 gene product is required for T4-induced host mRNA degradation, and the T4 $\Delta tk2$ mutant showed slower degradation of host

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¹Corresponding author: Department of Microbiology, School of Medicine, Dokkyo Medical University, 880 Kitakobayashi, Mibu-machi, Shimotsuga-gun, Tochigi 321-0293, Japan. E-mail: otsukay@dokkyomed.ac.jp

mRNAs (Ueno and Yonesaki 2004). However, the effect of $\Delta tk2$ on host mRNA degradation was partial, which suggests that multiple mechanisms are utilized for T4-induced host mRNA degradation. In this study, we found that host mRNAs were partially stabilized, that a decay intermediate rapidly accumulated in cells infected with a $\Delta(39-56)_6$ mutant lacking eight consecutive genes, and that the causal gene was *srd* (similarity with *rpoD*) encoding a 29-kDa protein. This gene was named *srd* because it partly shares sequence similarity with *E. coli* RNA polymerase sigma 70; Mosig *et al.* (1998) reported that exogenous expression of Srd in *E. coli* cells resulted in extremely slow growth and filamentation of cells. However, the function and the role of Srd in T4-infected cells remained unclear.

RNase E participates in maturation of transfer RNAs (tRNAs), processing of ribosomal RNAs (rRNAs), and turnover of bulk mRNAs in *E. coli* (Mudd *et al.* 1990b; Mackie 2013). It roughly consists of two domains, an N-terminal (1–529 aa) catalytic half (NTH) and a C-terminal (530–1036 aa) scaffold half (CTH); the former has a catalytic domain for RNase, and the latter is required for the targeting to the inner membrane, the interaction with RNA, and the binding of polynucleotide phosphorylase, enolase, and RhlB RNA helicase to form a multi-enzyme complex called the RNA degradosome (Callaghan *et al.* 2005; Khemici *et al.* 2008; Tsai *et al.* 2012; Mackie 2013). RNase E cleaves mRNAs at internal sites either directly or in a 5'-end-dependent manner (Bouvier and Carpousis 2011). The latter requires the removal of a pyrophosphate from a triphosphate at the 5'-end of the transcript by RNA pyrophosphohydrolase (RppH) prior to RNase E cleavages. Hundreds of mRNAs are the targets of the 5'-end-dependent mRNA degradation pathway because the disruption of *rppH* results in the increase of their amounts or stabilities (Deana *et al.* 2008). On the other hand, many other mRNAs appear to be degraded by RNase E using another mechanism, the direct cleavage, independently from RppH (Clarke *et al.* 2014).

Here, we strongly suggest that T4 Srd associates with the N-terminal half of RNase E and stimulates both the 5'-end-dependent and -independent mRNA degradation activities of RNase E. Because T4 phage lacking *srd* exhibited reduced growth, the degradation of host mRNAs by RNase E activity that Srd stimulates after infection is required for efficient phage growth.

Materials and Methods

Phages and bacterial strains

Wild-type bacteriophage T4 is T4D. A $\Delta(39-56)_6$ phage was kindly provided by H. Takahashi (University of Tokyo). Δsrd , $\Delta motB.2$, $\Delta dda-dda.1$, $\Delta dexA.1-dexA.2$, $\Delta modA$, and $\Delta dexA$ mutant phages were constructed using the insertion/substitution system (Selick *et al.* 1988). Briefly, a DNA fragment was amplified by polymerase chain reaction (PCR) using T4 DNA as the template and primers 1 and 2 (Supporting Information, Table S1). Next, the amplified fragment was used for a

second PCR as the primer together with primer 3 using T4 DNA as the template. Consequently, the resulting fragment contained 5' and 3' flanking regions of target gene(s) that were deleted. This DNA fragment was inserted into pBSPL0+, and the mutant sequence was transferred into the T4 genome via homologous recombination between plasmid and T4 DNA.

E. coli K-12 strains MH1 (*sup⁰ araD139 hsdR $\Delta lacX74 rpsL$*), TY0807 (MH1 *araD⁺*) (Koga *et al.* 2011), and YT10 (MH1 *zce726::Tn10*) were used as wild types. YT10 or YT20 (YT10 *ams1*) was constructed by T4 GT7 phage transduction (Wilson *et al.* 1979) of the tetracycline-resistance marker from GW10 (Wachi *et al.* 1997) or by GT7 phage transduction of the tetracycline-resistance marker together with a temperature-sensitive mutation of *ams1* from GW20 (Wachi *et al.* 1997). YT007 (MH1 $\Delta rppH::kan$) was also constructed by GT7 phage transduction of the kanamycin-resistance marker from JW2798 (NIG). The kanamycin-resistance cassette of YT007 was removed by yeast Flp recombinase expressed from pCP20 to construct TY1005 (MH1 $\Delta rppH$) (Cherepanov and Wackernagel 1995). $\Delta lpp::kan$ of JW1667 (NIG) was transferred into YT10, YT20, or TY1005 by GT7 phage transduction to construct TY1001 (MH1 *zce726::Tn10 $\Delta lpp::kan$*), TY1002 (MH1 *zce726::Tn10 ams1 $\Delta lpp::kan$*), or TY1006 (MH1 $\Delta rppH $\Delta lpp::kan$$), respectively. TY1007 (MH1 *rne-FLAG-cat*) and TY1008 (MH1 *rne598-FLAG-cat*) were also constructed by GT7 phage transduction of the chloramphenicol-resistance marker from TM338 (W3110 *mlc rne-FLAG-cat*) or TM529 (W3110 *rne598-FLAG-cat*), which were kindly provided by H. Aiba (Nagoya University).

Plasmids

To clone T4 *srd*, a 785-bp DNA fragment (11039–11803 of GenBank accession no. NC_000866) was amplified by PCR using T4 DNA as the template and primers 5'-ACGCGTC-GACGTAAGATGTGAGAA and 5'-ACGCGTCGACTTATCCTCG-GATAAG and digested with *SalI*. The resulting fragment was inserted into the *SalI* site of pBAD18 or pBAD33 (Guzman *et al.* 1995) to generate pBAD18-*srd* or pBAD33-*srd*, respectively. To construct pQE80L-*srd*-His, the DNA fragment encoding Srd with a His-tag at its C terminus was amplified by PCR with T4 DNA as the template and the primers 5'-GACTCCGGAA TTCCGGGTAAGATGTGAGAA and 5'-GACCCAAGCTTGGG TTAGTGGTGATGGTGATGATGTCCTCGGATA, digested with *EcoRI* and *HindIII* and ligated into the corresponding sites of pQE80L (Qiagen). To generate pBS*lpp*(T), the primer 1 (5'-CTATTACGCCAGCTGGCGAAAAAATGGCGACAATGTGC) and the primer 2 (5'-GGCCGATTCATTAATGCAGCTGGC) were first phosphorylated using T4 kinase with ATP. The phosphorylated primer 1 and the primer 3 (5'-CGCGCCCAGTACCAGTTTAGTAGCTTCCACACAACATACGAGCCGGAAGCAT) were used for PCR with *E. coli* MG1655 DNA as a template. The PCR-amplified fragment was used as the primer for the second PCR with the phosphorylated primer 2 and pBluescript II KS (+) as the template. Finally, the resulting fragment was ligated into the *PvuII* site of pBluescript II KS (+).

Determination of the deleted region in the $\Delta(39-56)_6$ phage

Two primers—5'-TCAACGCCATCTTCCAATCCAT located in *motB.1* and 5'-ATCTTGACAGATGTTGAACAGT located in *modB*—were used for PCR. When $\Delta(39-56)_6$ phage DNA was used as a template, only a prominent band corresponding to 1.2 kb was produced, although 5.8-kb DNA was amplified with wild-type phage DNA as a template. The 1.2-kb DNA amplified with $\Delta(39-56)_6$ phage DNA was recovered from the gel and sequenced. The sequence context showed that the nucleotide at 7744 in the T4 genome was connected with that at 12,364, indicating that $\Delta(39-56)_6$ lacks a 4619-bp sequence.

RNA purification, Northern blot, and primer extension analyses

Isolation of total RNA and Northern blot analyses were carried out as described (Kai *et al.* 1996; Ueno and Yonesaki 2004). Cells were infected with T4 phage at a multiplicity of infection (m.o.i.) of 7 for examining the half-lives of mRNAs after infection. To measure half-lives of mRNAs in the absence of T4 infection, rifampicin was added to a final concentration of 500 $\mu\text{g/ml}$. Total RNAs extracted were electrophoresed through a 5% polyacrylamide gel containing 7 M urea, followed by Northern blotting with probes for *lpp ompA* or *srd*. Oligo probes for *srd* and *lpp* were 5'-³²P-ACGCGTCGACTTATCCTCGGATAAG and 5'-³²P-TTACTTGCGGTATTTAGTAG, respectively. Northern blot analyses were performed more than twice independently and a representative result is shown in Figure 1, Figure 2, Figure 3, Figure 4, and Figure 6. The relative amount of each RNA was normalized to 5S rRNA. Primer extension analysis for *lpp* RNA was performed using 5'-³²P-GCATTGCGTTCACGTCGTTGCTCA as the primer.

Immunoprecipitation, pull-down, and Western blot analyses

TY1007 or TY1008 cells harboring pQE80L-*srd*-His or pQ-*orf2-95* encoding His-tagged IscR (Otsuka *et al.* 2010) were grown at 30° in 200 ml of LB medium containing 50 $\mu\text{g/ml}$ ampicillin until the OD₆₀₀ reached 0.6 and treated with 2 mM IPTG for 2 hr to induce His-tagged Srd or 20 μM IPTG for 1 hr to induce His-tagged IscR. Cells were harvested and suspended in 1.5 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM DTT, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, and 1 \times protease inhibitor cocktail). One hundred microliters of the cell mixture was kept as a whole-cell extract. After sonication, the lysate was centrifuged at 20,400 \times g for 20 min and separated into the supernatant as a soluble fraction and the pellet as an insoluble fraction. The pellet was suspended with 1.3 ml of TBS buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM DTT). The soluble fraction (1.3 ml) was treated with 20 $\mu\text{g/ml}$ RNase A for 30 min at 25°, and then 0.6 ml was mixed with 20 μl of Ni-NTA Superflow agarose beads (Qiagen) or 20 μl of anti-FLAG M2 affinity agarose (Sigma-Aldrich) by end-over-end rotation overnight at 4°. The beads were washed four times with 0.6 ml of TBS

buffer for anti-FLAG M2 agarose or TBS buffer containing 20 mM imidazole for Ni-NTA agarose, and then 45 μl of sample loading buffer was added to the beads. After boiling the samples, bound proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [10% gel for full-length RNase E-FLAG, 12% gel for RNase E(1-598)-FLAG, or 15% gel for Srd-His and His-IscR] and electroblotted onto Immuno-Blot PVDF membrane (Bio-Rad). Western blot analysis was performed as previously described (Koga *et al.* 2011). Pull-down and immunoprecipitation analyses were performed at least three times, and a representative result is shown in Figure 5.

Data availability

File S1 contains Table S1 and Figures S1, S2, S3, S4, S5, and S6.

Results

Identification of a phage factor involved in T4-induced host mRNA degradation

To identify factor(s) involved in T4-induced host mRNA degradation, we examined several T4 deletion mutants that lack multiple genes in various genomic regions and found that the $\Delta(39-56)_6$ mutant affected the degradation of host mRNAs. MH1 (wild-type) cells were grown until the OD₆₀₀ reached 0.5, and total RNAs were extracted from cells at appropriate times after infection and examined by Northern blot analysis. In cells infected with wild-type phage, the representative stable mRNAs *lpp* and *ompA* were rapidly degraded after infection with half-lives of 2.4 and 1.4 min, respectively (Figure 1, B and C). When infected with the $\Delta(39-56)_6$ mutant, the half-lives of *lpp* and *ompA* mRNAs were increased to 4.6 and 3.1 min, respectively. In addition, a decay intermediate of *lpp* mRNA was remarkably accumulated in $\Delta(39-56)_6$ -infected cells. This result suggested that a gene lacking in $\Delta(39-56)_6$ was involved in T4-induced host mRNA degradation. The $\Delta(39-56)_6$ mutant was originally isolated by Homyk and Weil in 1974 (Homyk and Weil 1974), but the exact region deleted in the genome of this phage was not reported. We mapped the deletion by PCR and DNA sequencing and found it lacking a 4619-bp DNA sequence from nucleotides 7745 to 12,363 in the T4 genome, resulting in the lack of all or a part in eight ORFs (Figure 1A). To identify a gene responsible for T4-induced host mRNA degradation, we constructed six mutants that lacked one or a few of these ORFs and examined the stability of *lpp* mRNA and the accumulation of the decay intermediate after T4 infection (Figure 1B and Figure S1). The deletion mutants (except Δsrd) exhibited little effect on decay of *lpp* mRNA and no accumulation of the decay intermediate. In contrast, *lpp* and *ompA* mRNAs were stabilized in Δsrd -infected cells, and their half-lives were 4.7 and 3.2 min, similar to those of $\Delta(39-56)_6$ -infected cells. In addition, the decay intermediate of *lpp* mRNA highly accumulated in Δsrd -infected cells. We also

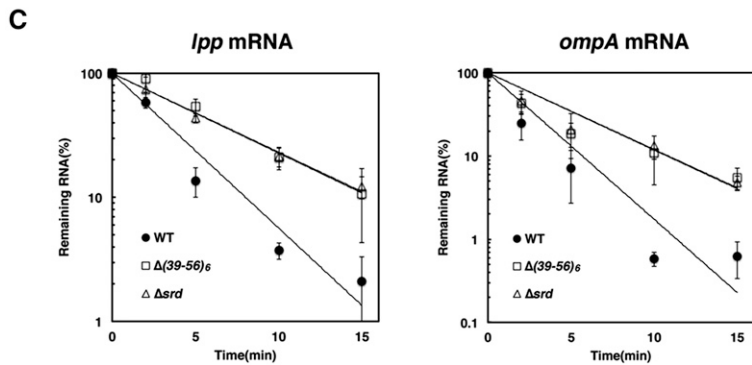
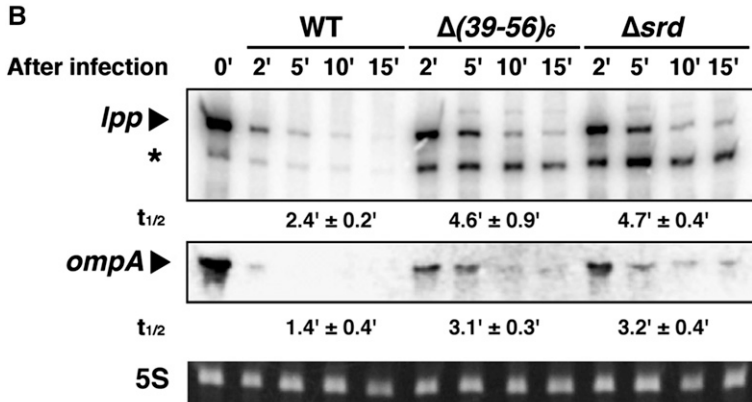
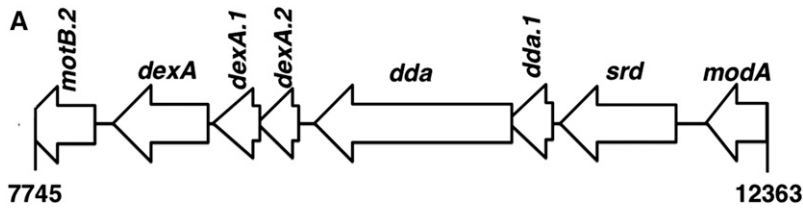


Figure 1 Involvement of *srd* in T4-induced host mRNA degradation. (A) Genetic organization of the chromosomal region deleted in the $\Delta(39-56)_6$ phage. (B) MH1 (wild-type) cells were grown in M9C medium (M9-glucose medium supplemented with 0.3% casamino acids, 1 μ g/ml thiamine, and 20 μ g/ml tryptophan) until the OD₆₀₀ reached 0.5 at 37° and infected with T4 wild-type, $\Delta(39-56)_6$, or Δsrd mutant. Total RNAs extracted at the indicated times were analyzed by Northern blotting with probes for *lpp* or *ompA*. Ethidium bromide-stained 5S rRNA is shown as a loading control. Arrowheads and an asterisk indicate each full-length mRNA and a decay intermediate of *lpp* mRNA, respectively. (C) Quantification analyses of full-length mRNAs in B were performed. The signal intensity was quantified using the Image J program, the signal obtained at 0 min was set to 100%, and the percentage of full-length mRNA remaining at each time point was plotted. Data points represent the mean \pm SD of triplicate measurements. The time required for a 50% reduction was taken as a half-life ($t_{1/2}$) of each mRNA shown below B.

examined the effect of *srd* on stabilities of typical unstable mRNAs, *rpsO*, *trxA*, and *rpsT*, and observed that *rpsO*(P1-t1) and *trxA* mRNAs were degraded after infection with wild-type phage slightly faster than in uninfected cells or after infection with the Δsrd mutant, although the stabilities of *rpsO*(P1-RIII) and *rpsT* mRNAs had no significant differences among uninfected cells, cells infected with T4 wild type, and the Δsrd mutant (Figure S2). These results strongly suggested that *srd* is involved in T4-induced host mRNA degradation.

Effect of Srd on decay of *lpp* and *ompA* mRNAs

To examine the effect of Srd on decay of *lpp* and *ompA* mRNAs in uninfected cells, we constructed a plasmid, pBAD18-*srd*, expressing Srd under the control of an arabinose-inducible

promoter. Total RNAs were extracted from cells harboring pBAD18-*srd* at 0, 5, 10, or 20 min after addition of arabinose and analyzed by Northern blotting. As seen in Figure 2A, the expression of *srd* mRNA was discernible at 5 min after addition of arabinose, and amounts of *lpp* and *ompA* mRNAs were reduced in contrast to the increase of *srd* mRNA. To further explore whether or not the reduction of these mRNAs is caused by the increase of decay rate, we measured stabilities of *lpp* and *ompA* mRNAs (Figure 2, B and C). At 10 min after Srd induction, a transcription inhibitor, rifampicin, was added to the culture, and total RNAs were extracted at appropriate times. In the absence of Srd, both *lpp* and *ompA* mRNAs were stable with half-lives of 27.1 and 12 min, respectively. In contrast, their half-lives were 11.7 min for *lpp* and 5.1 min for *ompA* in the presence of Srd,

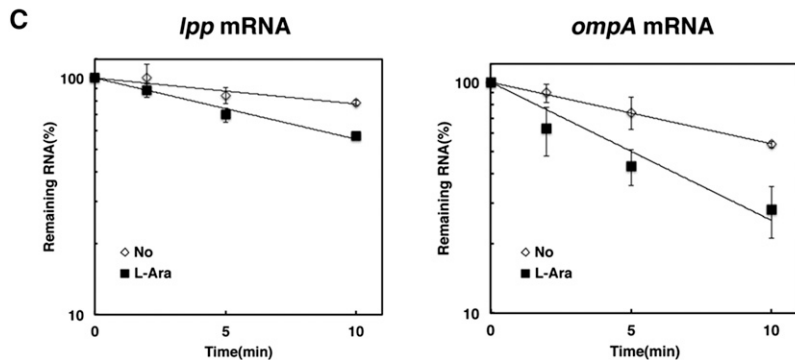
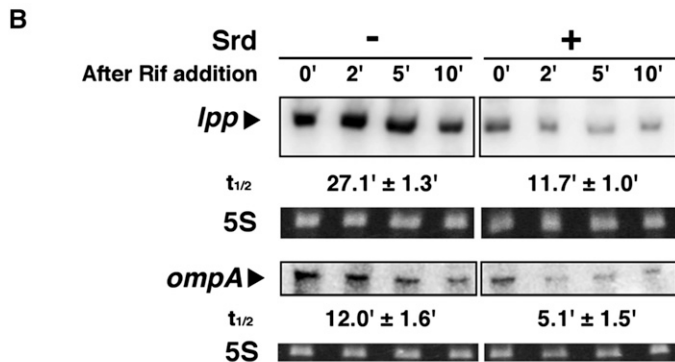
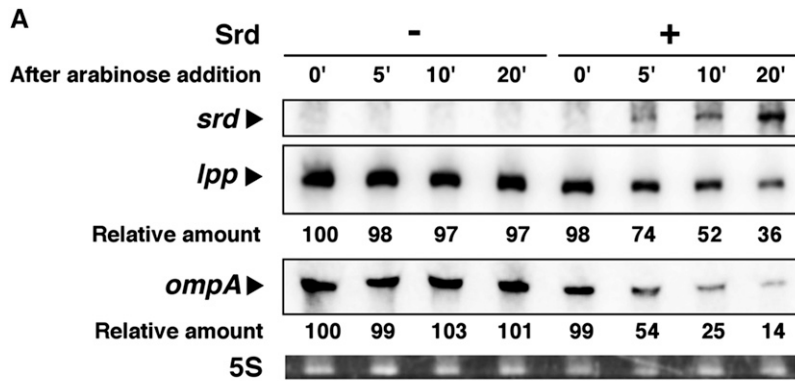


Figure 2 Destabilization of *lpp* and *ompA* mRNAs by exogenous expression of Srd. (A) TY0807 (wild-type) cells harboring pBAD18 (-) or pBAD18-*srd* (+) were treated with 0.15% arabinose when the OD₆₀₀ reached 0.5 at 30°. Total RNAs extracted at the indicated times were subjected to Northern blotting with probes for *srd*, *lpp*, or *ompA* mRNA. The intensity of the band at time 0 was set to 100%, and the relative intensity at each time is shown below. (B) TY0807 cells harboring pBAD18-*srd* were treated with (+) or without (-) 0.15% arabinose for 10 min when the OD₆₀₀ reached 0.5 at 30°. Total RNAs extracted at the indicated times after addition of the transcription inhibitor rifampicin were subjected to Northern blotting with probes for *lpp* or *ompA*. (C) Quantification analyses of full-length mRNAs in B were performed. Data points represent the mean ± SD of triplicate measurements.

indicating that their decay rates were increased approximately twofold after Srd induction. Taken together with the result of Figure 1B, Srd destabilizes *lpp* and *ompA* mRNAs.

Effect of Srd on 5'-end-independent cleavage activity of RNase E

The decay intermediate of *lpp* mRNA accumulated in Δ *srd*-infected cells (Figure 1B) would be attributable to the cleavage by RNase E because full-length *lpp* mRNA was considerably stabilized in RNase E-defective cells (Ueno and Yonesaki 2004). To confirm this possibility, we first examined the effect of RNase E on decay of *lpp* mRNA in Δ *srd*-infected cells. Wild-type or temperature-sensitive RNase E mutant (*ams1*) cells were grown at a permissive temperature (30°) until the OD₆₀₀ reached 0.3, and then the temperature was shifted to 44°, a nonpermissive temperature, for 30 min, followed

by infection with wild-type or Δ *srd* mutant. RNAs extracted from cells at appropriate times after infection were analyzed by Northern blotting. Full-length *lpp* mRNA was stabilized, and the decay intermediate was hardly detected in RNase E-defective cells infected with Δ *srd* mutant as well as wild-type phage (Figure 3, A and B). The cleavage site to generate this decay intermediate was determined by primer extension analysis, and its 5'-terminus was two nucleotides downstream of the start codon of *lpp* mRNA (AUGA↓AA) (Figure 3C and Figure S3). RNase E has no canonical target sequence for cleavage, but preferentially cleaves at regions that are single-stranded with AU-rich sequences. These facts clearly support the idea that RNase E generates this decay intermediate. Finally, we examined the effect of RppH on decay of *lpp* and *ompA* mRNAs stimulated by Srd (Figure 3, D and E). After infection with wild-type phage, both mRNAs were degraded in Δ *rppH* cells as fast as in wild-type cells, indicating

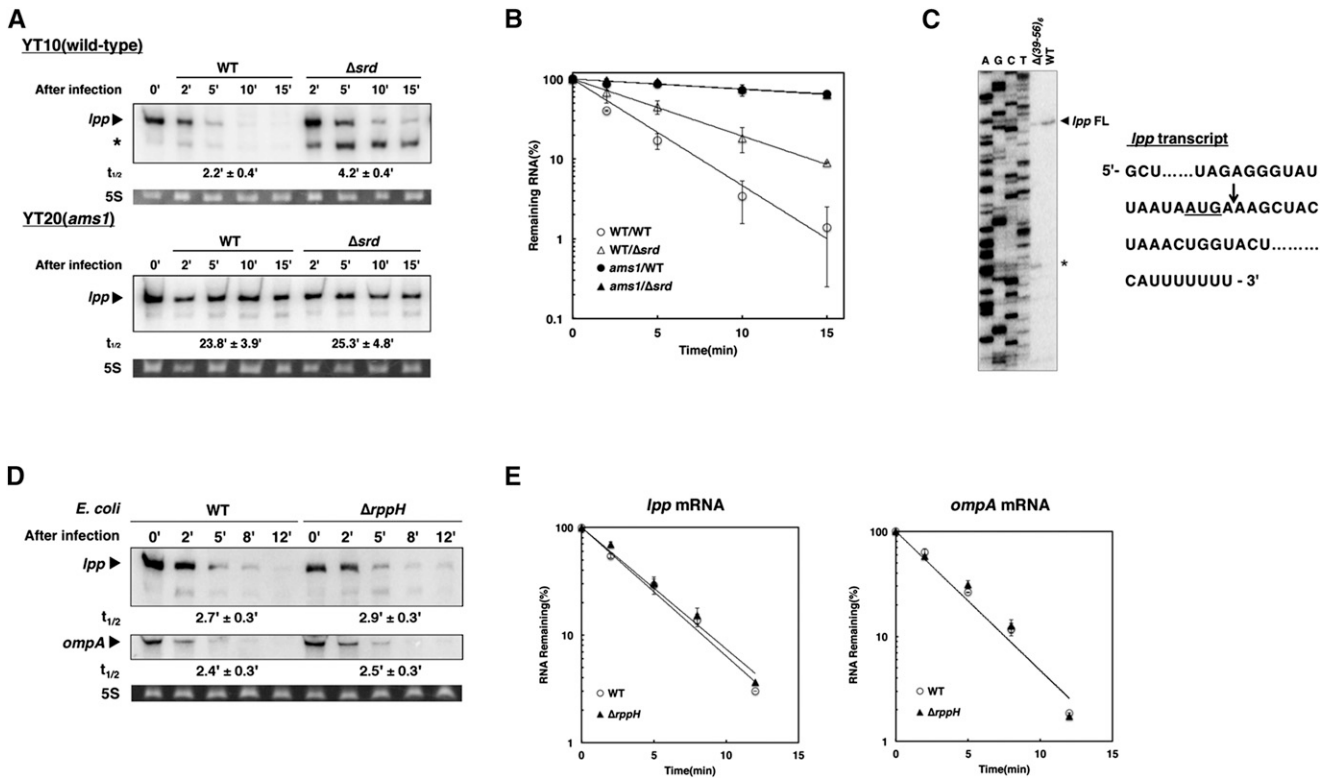


Figure 3 Stimulation of 5'-end-independent cleavage activity of RNase E by Srd. (A) YT10 (wild-type) or its derivative, YT20 (*ams1*), cells were grown in M9C medium until the OD₆₀₀ reached 0.3 at 30° and shifted to 44° for another 30 min and then infected with wild-type or Δsrd mutant. Total RNAs extracted at the indicated times were analyzed by Northern blotting with a probe for *lpp*. An arrowhead and an asterisk indicate the full-length *lpp* mRNA and its decay intermediate, respectively. (B) Quantification analysis of *lpp* mRNA in A was performed. Data points represent the mean \pm SD of triplicate measurements. (C) Five micrograms of total RNAs extracted from MH1 cells infected with wild-type or $\Delta(39-56)_6$ mutant were used for primer extension analysis as described in *Materials and Methods*. "FL" or the asterisk denotes the full-length *lpp* mRNA or its decay intermediate. A set of sequence ladders for *lpp* obtained by dideoxy-sequencing with the same primer was run in parallel. The RNA sequence around the SD region and the start codon (underlined text) of *lpp* mRNA are shown at the right, and the arrow indicates the cleavage site generating the decay intermediate. (D) MH1 (WT) or TY1005 ($\Delta rppH$) cells were grown in M9C medium until the OD₆₀₀ reached 0.5 at 37°, and total RNAs were extracted at the indicated times after infection with wild type and then analyzed by Northern blotting with probes for *lpp* or *ompA*. (E) Quantification analyses of full-length mRNAs in D were performed. Data points represent the mean \pm SD of duplicate measurements.

that RppH is not related to the degradation of their mRNAs. From these results, RNase E degrades these mRNAs through an RppH-independent manner in the presence of Srd.

Effect of Srd on 5'-end-dependent cleavage activity of RNase E

The decay intermediate of *lpp* mRNA should be processively degraded by RNase E in the presence of Srd because it is not observed in cells infected with wild-type phage. To investigate the effect of Srd on the decay intermediate of *lpp* mRNA, we constructed a plasmid, pBS*lpp*(T), expressing the truncated *lpp* RNA with a sequence identical to the decay intermediate of *lpp* mRNA from the *lac* promoter of pBluescript II KS (+) (Figure S3). First, we characterized the decay of *lpp*(T) RNA in the absence of Srd. At 44°, *lpp*(T) RNA had a half-life as short as 1.4 min in $\Delta lpp::kan$ cells expressing wild-type RNase E, while it was more stable with a half-life of 9.5 min in $\Delta lpp::kan$ *ams1* cells defective in RNase E activity (Figure S4, A and B). *lpp*(T) RNA should have a triphosphate at the 5'-end, which differs from a decay intermediate of *lpp* mRNA

with a 5'-monophosphate end. Therefore, the degradation of *lpp*(T) RNA may be dependent on RppH. To address this issue, we examined the effect of RppH on stability of *lpp*(T) RNA (Figure S4, C and D). The half-life of *lpp*(T) RNA in wild-type or $\Delta rppH$ cells was 2.8 or 7.1 min, respectively. Taken together, these results show that *lpp*(T) RNA is degraded by RNase E through an RppH-dependent pathway under normal conditions.

The effect of Srd on decay of *lpp*(T) RNA was examined by measuring the amount of *lpp*(T) RNA with or without induction of Srd (Figure 4A), and we found that the amount was considerably reduced after induction of Srd for 10 min. Furthermore, we checked the stability of *lpp*(T) RNA (Figure 4, B and C). The half-life with or without induction of Srd was 1.3 or 2.9 min, respectively, clearly demonstrating that Srd stimulates the degradation of *lpp*(T) RNA. Next, we confirmed that Srd stimulates the RNase E-dependent decay of *lpp*(T) after T4 infection (Figure 4, D and E). The half-life of *lpp*(T) RNA in the presence or absence of *srd* was 2.9 or 7.8 min, respectively, at 30°, while it was 7.7 or 9.4 min at 44°. Finally,

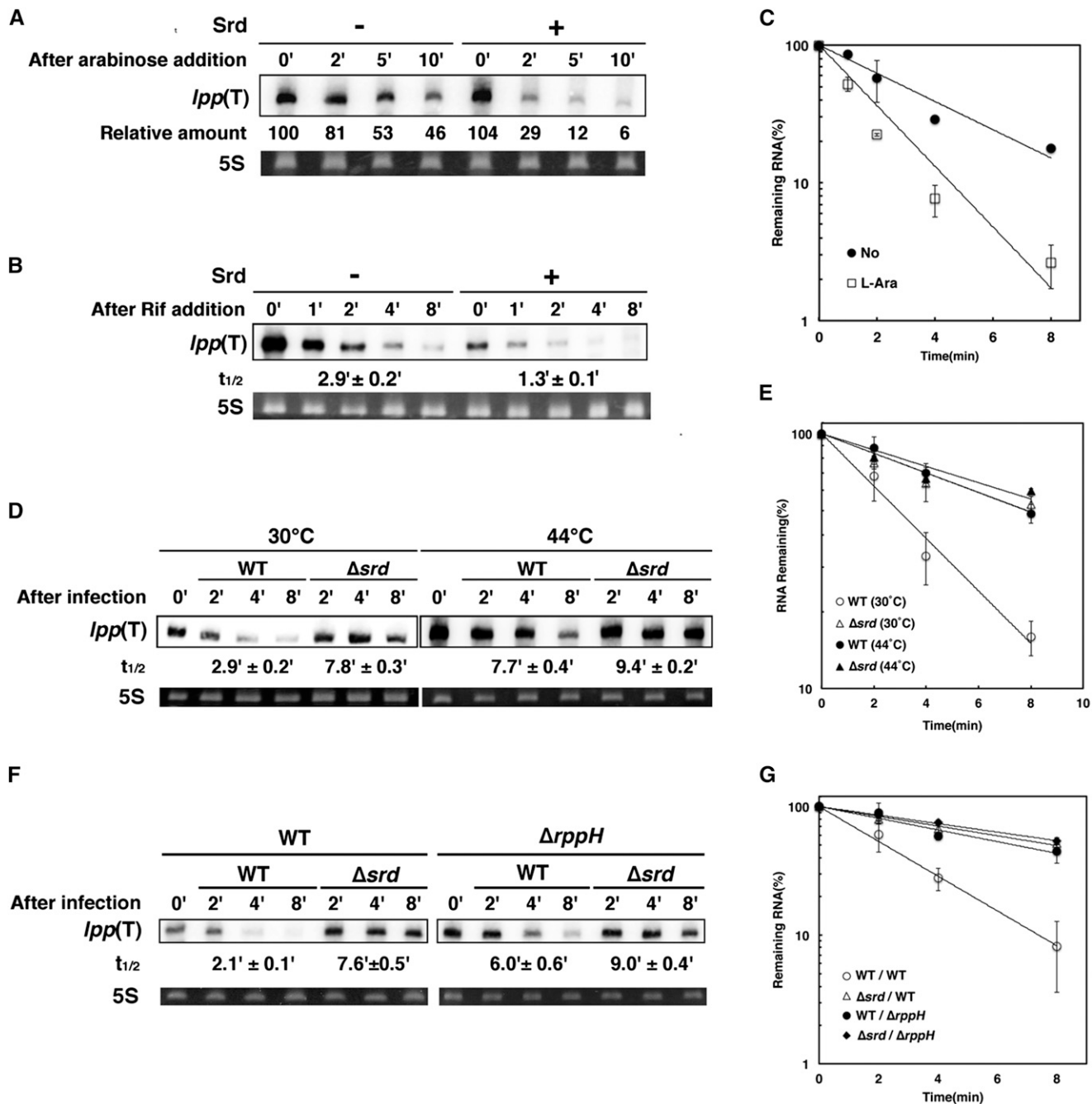


Figure 4 Stimulation of 5'-end-dependent cleavage activity of RNase E by Srd. (A) TY1001 ($\Delta lpp::kan$) cells harboring pBS/*lpp(T)* plus either pBAD33 (-) or pBAD33-*srd* (+) were grown at 30° until the OD₆₀₀ reached 0.5 and supplemented with 1 mM IPTG. At 10 min after IPTG addition, cells were treated with 0.2% arabinose, and then total RNAs extracted at the indicated times were subjected to Northern blotting with a probe for *lpp*. The intensity of band at time 0 was set to 100%, and relative intensity at each time is shown below. (B) TY1001 cells harboring pBS/*lpp(T)* and pBAD33-*srd* were grown at 30° until the OD₆₀₀ reached 0.5 and treated with 1 mM IPTG. At 10 min after IPTG addition, cells were treated with (+) or without (-) 0.05% arabinose for another 10 min. Total RNAs extracted at the indicated times after addition of rifampicin were subjected to Northern blotting with a probe for *lpp*. (C) Quantification analysis of *lpp(T)* RNA in B was performed. Data points represent the mean \pm SD of triplicate measurements. (D) TY1002 ($\Delta lpp::kan$ *ams1*) cells harboring pBS/*lpp(T)* were grown at 30° until the OD₆₀₀ reached 0.3, divided into two aliquots, and incubated for 25 min at 30° or 44°, respectively. Then the cells were treated with 1 mM IPTG for 10 min and further divided into two aliquots followed by infection with wild-type or Δsrd mutant. Ten micrograms of total RNAs extracted at the indicated times after infection was subjected to Northern blotting with a probe for *lpp*. (E) Quantification analysis of *lpp(T)* RNA in D was performed. Data points represent the mean \pm SD of five time measurements. (F) TY1001 ($\Delta lpp::kan$) or TY1006 ($\Delta lpp::kan$ $\Delta rppH$) cells harboring pBS/*lpp(T)* were grown at 30° until the OD₆₀₀ reached 0.5. Then the cells were treated with 1 mM IPTG for 10 min followed by infection with wild-type or Δsrd mutant. Ten micrograms of total RNAs extracted at the indicated times after infection was subjected to Northern blotting with a probe for *lpp*. (G) Quantification analysis of full-length mRNAs in F was performed. Data points represent the mean \pm SD of triplicate measurements.

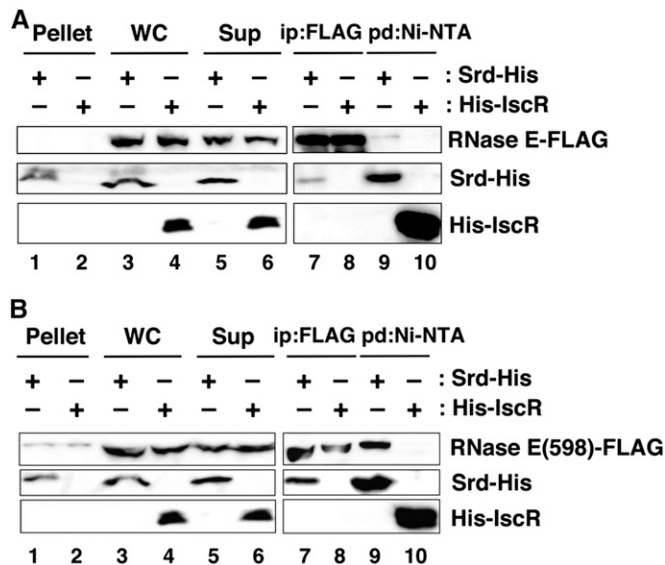


Figure 5 Physical association between RNase E and Srd *in vivo*. (A) TY1007 (*rne-FLAG-cat*) cells or (B) TY1008 (*rne598-FLAG-cat*) cells harboring either pQE80L-*srd*-His or pQ-orf2-95 were grown at 30° until the OD₆₀₀ reached 0.6, and IPTG was added to induce Srd-His or His-IscR. Preparation of cell extracts and fractions, immunoprecipitation with anti-FLAG M2 agarose beads, and pull-down with Ni-NTA beads were carried out as described in *Materials and Methods*. A total of 0.33% of whole-cell extract (WC, lanes 3 and 4), the insoluble fraction (Pellet, lanes 1 and 2), and the soluble fraction (Sup, lanes 5 and 6), and 33.3% of the bound fractions (lanes 7–10) was analyzed by Western blotting with antibodies against FLAG-tag (top) and His-tag (middle and bottom).

we examined the effect of RppH on decay of *lpp*(T) RNA after T4 infection. When wild-type phage infected, *lpp*(T) RNA was significantly stabilized in Δ *rppH* cells (6.0 min), compared with wild-type cells (2.1 min) (Figure 4, F and G). These results indicate that T4 Srd, RNase E, and RppH are required for the rapid degradation of *lpp*(T) RNA after T4 infection. Taken together with all the results, Srd should stimulate both 5'-end-independent and 5'-end-dependent cleavage activities of RNase E either directly or indirectly.

Association of Srd and RNase E *in vivo*

The above results implied that Srd physically associated with RNase E. Hence, we carried out immunoprecipitation and pull-down analyses to examine their interaction *in vivo*. For these experiments, we used TY1007 cells in which the chromosomal *rne* was replaced with *rne-FLAG* encoding FLAG-tagged RNase E. Extracts prepared from TY1007 cells expressing His-tagged Srd or His-tagged IscR as a control were used for immunoprecipitation with FLAG antibody or pull-down with Ni-NTA. Before immunoprecipitation and pull-down analyses, we checked how much RNase E was solubilized because RNase E is membrane-associated. As seen in Figure 5A (lanes 1–6), the majority of RNase E was present in the soluble fraction under our experimental conditions. In the immunoprecipitation experiment, Srd-His was efficiently recovered together with RNase E-FLAG (lane 7), but His-IscR was not (lane 8). In the reciprocal pull-down experiment,

RNase E-FLAG was precipitated together with Srd-His (lane 9), but not with His-IscR (lane 10). These results suggested the physical association of Srd and RNase E *in vivo*.

Dispensability of RNase E CTH for mRNA degradation stimulated by Srd

RNase E is composed of NTH (1–529) and CTH (530–1036) (Callaghan *et al.* 2005; Mackie 2013). We investigated whether or not CTH was indispensable for T4-induced host mRNA degradation. TY1008 cells expressing FLAG-tagged RNase E (1–598) containing the catalytic domain (1–529) and the membrane target sequence (segment A, 568–582) from the genome were infected with wild-type or Δ *srd* mutant, and total RNAs were extracted at appropriate times for Northern blot analysis. Consistent with the previous report (Ueno and Yonesaki 2004), RNase E lacking the C-terminal scaffold domain degraded full-length *lpp* mRNA more slowly than did normal RNase E, and its half-life was 5.0 min (compare to 2.4 min in Figure 1B) when infecting with wild-type phage (Figure 6, A and B). When infecting with the Δ *srd* mutant, full-length *lpp* mRNA was more stabilized with a half-life of 9.2 min, and the decay intermediate also accumulated. This result paralleled the result in Figure 1B, suggesting that the C-terminal scaffold domain (599–1036) is not necessary for *E. coli* mRNA degradation stimulated by Srd.

Prompted by the above result, we examined the activity of the N-terminal half (1–598) in association with Srd. As seen in Figure 5B, most of RNase E(598)-FLAG was present in the soluble fraction (lanes 1–6), like an RNase E-FLAG. Srd-His was precipitated by FLAG antibody together with RNase E (598)-FLAG (lane 7), but His-IscR was not (lane 8). In the pull-down experiments with Ni-NTA beads, RNase E(598)-FLAG was recovered in the bound fraction together with Srd-His (lane 9) but hardly with His-IscR (lane 10), indicating the association of Srd and the N-terminal half of RNase E containing the catalytic domain and the membrane target sequence *in vivo*.

Effect of *srd* on growth of T4 phage

Finally, we investigated the effect of *srd* on T4 growth. Wild-type or Δ *srd* mutant was spread on plates seeded with MH1 (wild-type) cells as an indicator (Figure 7A). The Δ *srd* mutant formed smaller plaques than those of wild type. We also confirmed the stimulatory effect of *srd* on propagation of T4 phage by measuring burst size (Figure 7B). Consistently with the observation of plaque size, the burst size of the Δ *srd* mutant was 24.4 ± 1.9 , while that of wild type was 86.9 ± 4.8 . These results indicate that *srd* is required for efficient growth of T4 phage.

Discussion

In this study, we demonstrated that T4 Srd is involved in phage-induced host mRNA degradation in which *E. coli* RNase E plays a central role. RNase E participates in the turnover of bulk mRNAs as well as the processing of tRNAs and

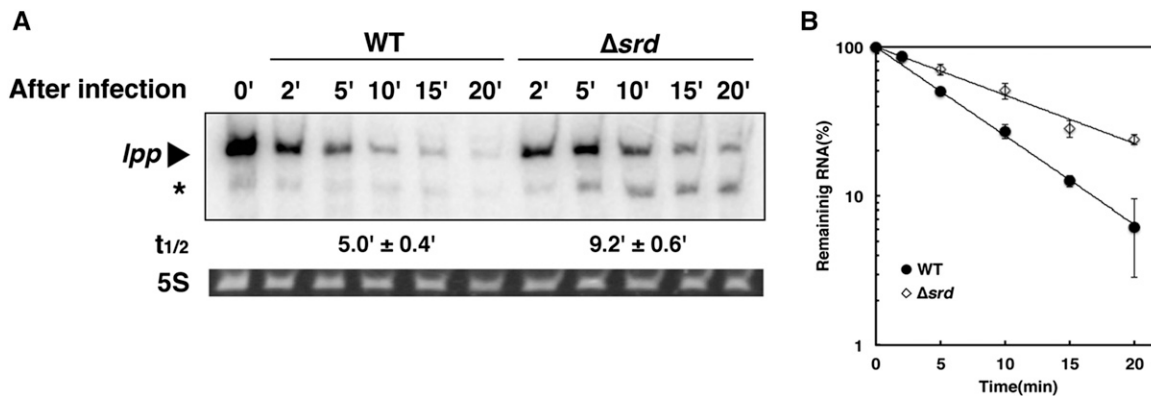


Figure 6 Effect of CTH of RNase E on T4-induced host mRNA degradation. (A) TY1008 (*rne598-FLAG-cat*) cells were grown at 30° in M9C medium until the OD₆₀₀ reached 0.5, divided into two aliquots, and infected with wild-type or Δ *srd* mutant. Total RNAs extracted at the indicated times were subjected to Northern blotting with a probe for *lpp*. (B) Quantification analysis of *lpp* mRNA in A was performed. Data points represent the mean \pm SD of duplicate measurements.

rRNAs in *E. coli* and many other proteobacteria (Ono and Kuwano 1979; Jain *et al.* 2002; Carpousis 2007; Tsai *et al.* 2012). Cleavage of mRNA by RNase E can occur via two pathways: 5'-end monophosphate-dependent and 5'-end independent (so-called "direct entry") (Anupama *et al.* 2011; Bouvier and Carpousis 2011; Garrey and Mackie 2011). RppH is necessary for the former case (Celesnik *et al.* 2007; Deana *et al.* 2008; Bouvier and Carpousis 2011). RppH removes a pyrophosphate from a 5'-end triphosphate of mRNA and then RNase E cleaves RNA due to the preference of RNase E for 5'-monophosphorylated RNA. Hundreds of mRNAs are reported to be the targets of RppH, such as *rpsT*, *yeiP*, and *trxB* (Deana *et al.* 2008; Richards *et al.* 2012). Our study showed that the plasmid-borne *lpp*(T) RNA was degraded by RNase E in an RppH-dependent fashion (Figure S4), and that Srd stimulated this activity (Figure 4). In the 5'-end-independent pathway, RNase E first triggers the degradation of mRNA by cleavage at an internal site to generate an RNA fragment with a monophosphate at 5'-end, and the sequential degradation by RNase E follows in a 5'-end-dependent fashion. *E. coli* stable mRNAs *ompA* and *lpp* are known as examples for this pathway (Emory *et al.* 1992; Ow *et al.* 2003; Deana *et al.* 2008). In fact, after T4 infection, *ompA* and *lpp* mRNAs were degraded in Δ *rppH* cells as fast as those in wild-type cells (Figure 3, D and E). Therefore, Srd could stimulate 5'-end-independent cleavage activity of RNase E. Taken together with all the analyses of *lpp*, *ompA* and *lpp*(T) mRNAs, we conclude that T4 Srd stimulates both 5'-end-dependent and independent cleavage activities of RNase E either directly or indirectly.

The NTH (1–529) of RNase E is necessary and sufficient for cleavage and forms a tetrameric assembly of RNase E (Mackie 2013). The CTH (530–1036) acts mainly as a scaffold for RNA degradosome assembly and recruits its core constituents: polynucleotide phosphorylase, enolase, and RhlB RNA helicase (Carpousis 2007; Góna *et al.* 2012). We demonstrated that T4-induced host mRNA degradation occurred in the absence of the C-terminal half (599–1036)

(Figure 6). Furthermore, immunoprecipitation and pull-down experiments showed that Srd was physically associated with the N-terminal half (1–598) of RNase E. Considering that the amount of RNase E did not change after T4 infection (Figure S5), Srd is strongly suggested to accelerate host mRNA degradation by stimulation of catalytic activity of RNase E. The N-terminal half (1–598) consists of two RNase H-like domains, an S1 domain, a 5' sensor domain, a DNase I-like domain, a Zn-link domain, a small domain, and the membrane target sequence (Mackie 2013). To identify which domain of RNase E interacts with Srd may help toward understanding the stimulatory mechanism by Srd. Furthermore, it would be also valuable to measure the kinetics of RNA cleavage by RNase E with or without Srd *in vitro*.

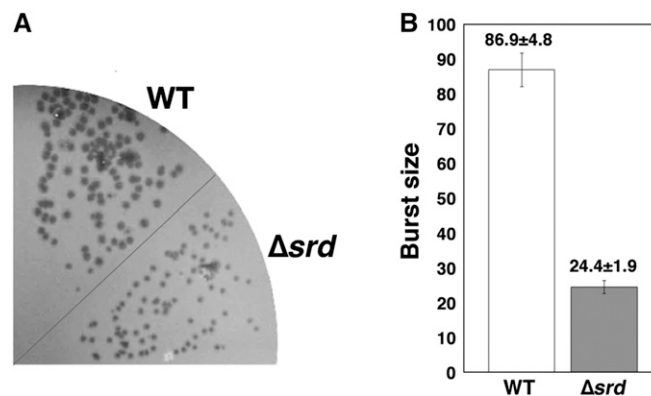


Figure 7 Growth of Δ *srd* mutant phage. (A) Equal number of wild-type and Δ *srd* mutant phage particles were plated onto a plate inoculated with MH1 (wild-type) cells and incubated at 37° overnight. (B) MH1 cells were grown in M9C medium until the OD₆₀₀ reached 0.5 and infected with wild-type or Δ *srd* mutant at an m.o.i. of 0.1 at 37°. At 8 min, the cells were diluted 10⁴-fold with fresh M9C medium and further incubated for 70 min. After the cells were lysed with chloroform, the total number of progeny phage was determined by plating after an appropriate dilution with MH1 as an indicator. The burst size shown in the bar graph is the ratio of the number of progeny to the number of input phage particles. Each value indicates the mean and standard deviation obtained from three independent experiments.

To date, three *E. coli* factors to regulate RNase E activity, RraA, RraB, and ribosomal protein L4, have been identified, but all of them regulate RNase E activity negatively (Lee *et al.* 2003; Gao *et al.* 2006; Yeom *et al.* 2008a,b; Singh *et al.* 2009). Apart from these factors in *E. coli*, only one viral factor has been reported to regulate the degradation of mRNAs by RNase E. A protein kinase of bacteriophage T7 phosphorylates RNase E at its CTH and stabilizes a subset of RNase E substrates (Marchand *et al.* 2001). In this case, T7 protein kinase would not play a role in the shutoff of host gene expression because it regulates RNase E activity negatively. Therefore, T4 Srd would be the first example of a factor that stimulates RNase E activity and induces the degradation of host mRNAs after infection.

Three different classes of promoters—early, middle, and late—initiate T4 transcription (Miller *et al.* 2003). *srd* is located immediately downstream of an early promoter, which is supposed to be expressed at a very early time after T4 infection (Mathews 1994; Miller *et al.* 2003). Although our attempt for detecting endogenous expression of Srd was not successful (data not shown), we could detect endogenous *srd* mRNA by semiquantitative RT-PCR (Figure S6). This result indicates that the level of *srd* mRNA during infection is almost the same as that derived from pBAD18-*srd*. The observations that the Δ *srd* mutant forms smaller plaques (Figure 7A) and exhibits a much lower burst size (Figure 7B) in comparison to wild-type phage indicate that Srd is required for efficient growth of T4 phage and suggest that host mRNA degradation by Srd-stimulated RNase E activity should contribute to the transition of gene expression from host to T4 immediately after infection. However, the effect of *srd* on host mRNA degradation is partial (Figure 1B). Previously, T4 Δ *tk2* mutant showed slower degradation of host mRNAs (Ueno and Yonesaki 2004), and the effect of Δ *tk2* on host mRNA degradation was also partial. Considered together, T4 phage may employ multiple factors and mechanisms to sustain rapid degradation of host mRNAs.

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Rapid Degradation of Host mRNAs by Stimulation of RNase E Activity by Srd of Bacteriophage T4

Dan Qi, Abdulraheem M. Alawneh, Tetsuro Yonesaki, and Yuichi Otsuka

Table S1 Primers used for construction of deletion mutant phages in this study.

Phage mutant	Primer 1 (5'-3')	Primer 2 (5'-3')	Primer 3 (5'-3')
<i>ΔmodA</i>	GACTCCCAAGCTTGCGCC TTCTGCTATG	GGATGCATCGGTGCGTGCTTCTATTCGGA AGTAATGATACCAGCTGGAAGTG	ATCTTGACAGATGTTGAACA GT
<i>Δsrd</i>	ACGCGTCGACTCATCGTA AATTC	CGTTTAGAGCATACTCAGGCATCTGCCTGA AAAGAGGATATCCGCTAAATTG	ACGCGTCGACATTGAGGT AGTTG
<i>Δdda-dda.1</i>	ACGCGTCGACTTATCGTT TTGTTG	GCGATAGTTTACCGAGACAAAGACGGTGT CACCCGTGGTCGTTATGATGTG	ACGCGTCGACGTAAGATG TGAGAA
<i>ΔdexA.1-dexA.2</i>	ACGCGTCGACTTATCGTT TTGTTG	GGAAGCTATTCGTGGAAATAACTAAAGAG GTCAAATTCGTGATTTAGCGAGG	ACGCGTCGACCAATTGCA GGGAAT
<i>ΔdexA</i>	ACGCGTCGACTTAGTTTA AGGTA	GGGAAGTGGTGAAAAAGCAGCGGTTGGG TCTTGAAGATGCTCCATCAGAGG	ACGCGTCGACAAATTTGA GGAAG
<i>ΔmotB.2</i>	TCAACGCCATCTTCCAAT CCAT	GACGCTATAACAATACGCCGAGATCTTGG CGGGCATCAAGTAATGTTCCG	GTACCGGAATCCGATGA ATTAGTTTC

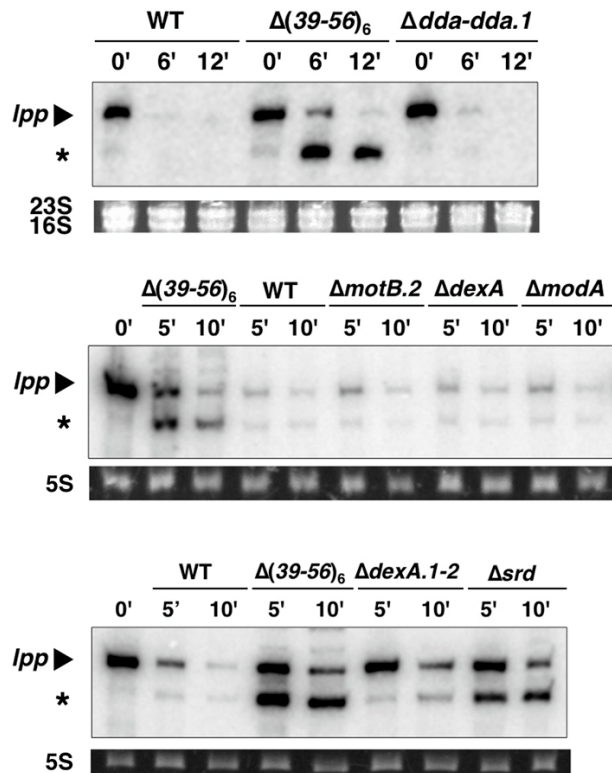
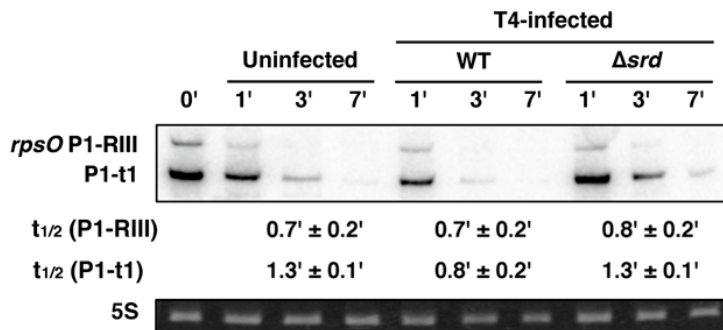
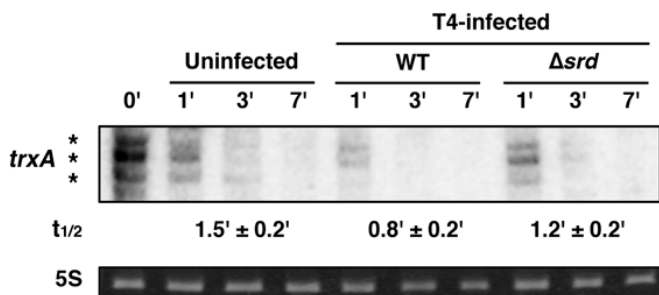


Figure S1 Effect of deleted genes on T4-induced host mRNA degradation. MH1 (wild-type) cells were grown in M9C medium until the OD_{600} reached 0.5 at 37°, and infected with T4 wild type, $\Delta(39-56)_6$, $\Delta dda-dda.1$, $\Delta motB.2$, $\Delta dexA$, $\Delta modA$, $\Delta dexA.1-dexA.2$ or Δsrd mutant phage. Total RNAs were extracted at the indicated times after infection and then analyzed by northern blotting with a probe for *lpp*. An arrowhead and an asterisk indicate full-length and a decay intermediate of *lpp* mRNA, respectively. Ethidium bromide-stained 5S rRNA or 23S/16S rRNA as a loading control is shown at the bottom of each panel. The Δsrd mutant demonstrated that *lpp* mRNA was stabilized and that the decay intermediate was strongly accumulated, as with $\Delta(39-56)_6$ mutant.

(A)



(B)



(C)

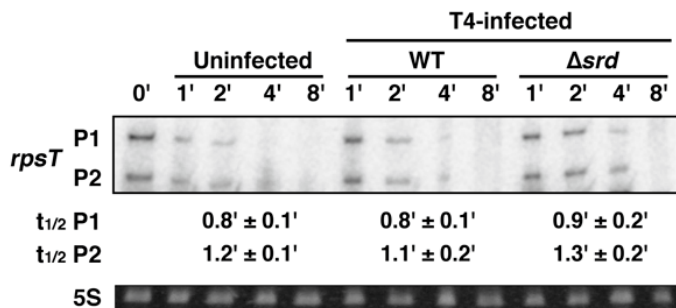


Figure S2 Effect of Srd on stabilities of unstable mRNAs. MH1 (wild-type) cells were grown in M9C medium until the OD_{600} reached 0.5 at 37°, and infected with wild type T4 or Δ *srd* mutant or treated with rifampicin at a final concentration of 250 μ g/ml. Total RNAs were extracted at the indicated times after infection or addition of rifampicin and then analyzed by northern blotting with an oligo-probe for *rpsO* (A, 5'-³²P- TTGCTTCAGTACTTAGAGAC), *trxA* (B, 5'-³²P- CTGTCGTCAGTCAGGTGAATAATTTTATCGCTC) or *rpsT* (C, 5'-³²P- AGAGCGACGGCTTGCGTTGTGCTTACGAGCCTTTTCAGACTGAATGGCGC). The *rpsO*(P1-t1) mRNA corresponds to the monocistronic *rpsO* transcript while the *rpsO*(P1-RIII) mRNA generates from the processing of the *rpsO-pnp* bicistronic transcript by RNase III (Hajnsdorf *et al.* 1994). Asterisks in the figure (B) indicate multiple *trxA* transcripts. Ethidium bromide-stained 5S rRNA as a loading control is shown at the bottom of each panel. Half-lives ($t_{1/2}$) with the mean \pm SD of duplicate measurements are shown below the figure. Like *lpp* and *ompA* mRNAs, *trxA* and *rpsO*(P1-t1) mRNAs were destabilized after infection with wild-type T4 and this destabilization was recovered by the deletion of *srd*.

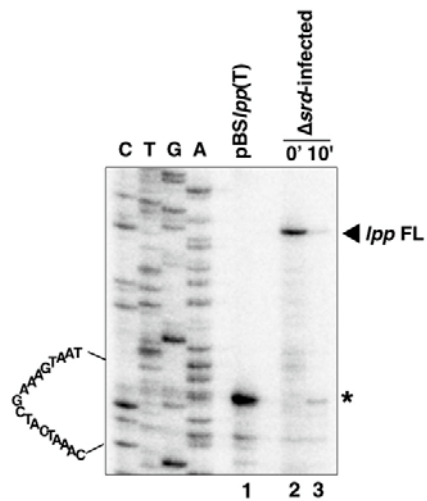


Figure S3 The 5' end of truncated *lpp* RNA driven from a pBS/*lpp*(T) plasmid. Five micrograms of total RNAs extracted from TY1001 ($\Delta lpp::kan$) cells harboring pBS/*lpp*(T) treated with 1 mM IPTG for 10 min (lane 1), from MH1(wild-type) cells at 0 min (lane 2) or at 10 min (lane 3) after infection with Δsrd mutant were used for primer extension analysis. FL or an asterisk denotes the full-length *lpp* mRNA or its decay intermediate, respectively. The 5' end of truncated *lpp* RNA expressed from pBS/*lpp*(T) was identical to that of the decay intermediate of *lpp* mRNA accumulated in Δsrd mutant-infected cells.

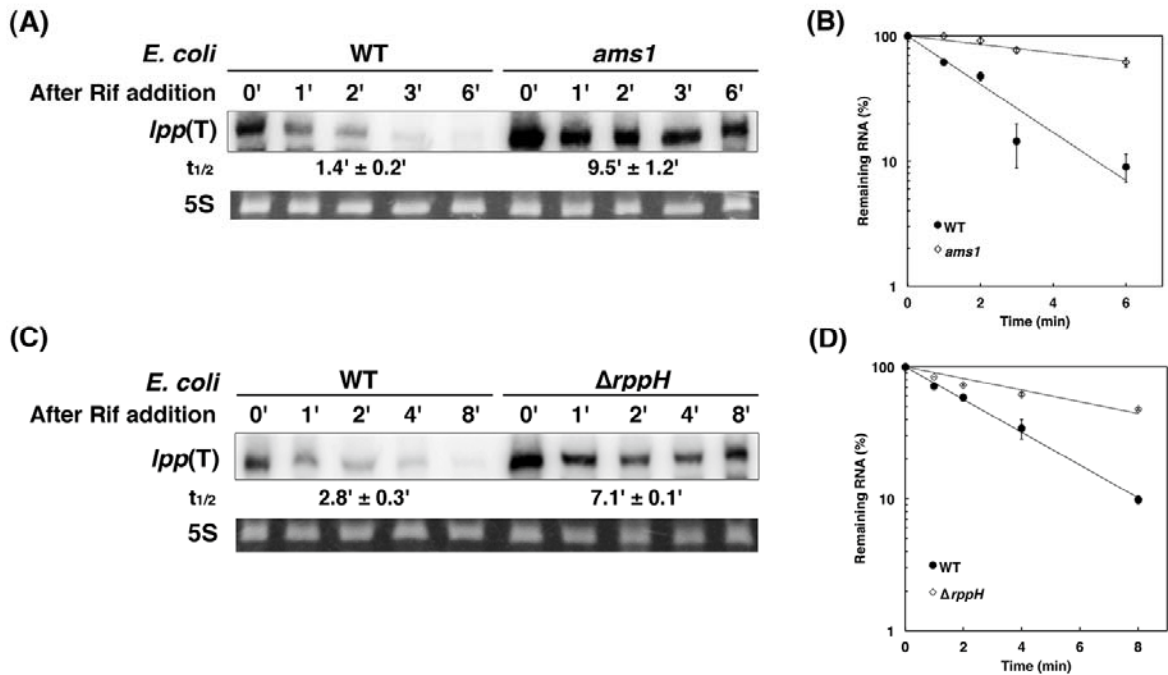


Figure S4 Degradation of *lpp(T)* RNA under normal growth condition. (A) TY1001 ($\Delta lpp::kan$) or TY1002 ($\Delta lpp::kan ams1$) cells harboring pBS*lpp(T)* were grown in LB medium until the OD_{600} reached 0.3 at 30° and shifted to 44° for another 30 min. After cells were treated with 1 mM IPTG for 10 min to induce expression of *lpp(T)* RNA, total RNAs extracted at the indicated times after addition of rifampicin were subjected to northern blotting with a probe for *lpp*. The RNA with a sequence identical to the truncated intermediate of *lpp* mRNA is labeled as *lpp(T)* (upper panel). (B) Quantification analysis of *lpp(T)* RNA in the figure (A) was performed. Data points represent the mean \pm SD of triplicate measurements. A half-life ($t_{1/2}$) of each mRNA is shown below the figure (A). (C) TY1001 or TY1006 ($\Delta lpp::kan \Delta rppH$) harboring pBS*lpp(T)* were treated with 1 mM IPTG for 10 min when the OD_{600} reached 0.5 at 30°. Total RNAs extracted at the indicated times after addition of rifampicin were subjected to northern blotting with a probe for *lpp*. (D) Quantification analysis of *lpp(T)* RNA in the figure (C) was performed. Data points represent the mean \pm SD of triplicate measurements. *lpp(T)* RNA was degraded by RNase E in RppH-dependent manner under normal growth condition.

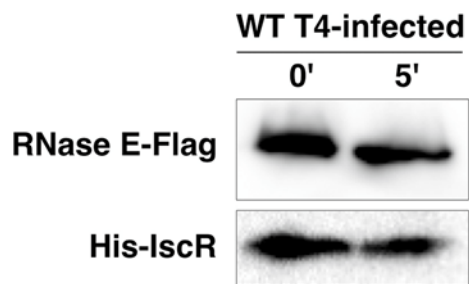


Figure S5 Amount of RNase E before and after T4 infection. TY1007 (*rne-FLAG-cat*) cells harboring pQ-orf2-95 were grown in LB medium until the OD_{600} reached 0.3 and treated with 0.02 mM IPTG for 30 min to induce expression of IscR. Cells were harvested immediately before (0 min) or 5 min after T4 infection. Equal amount of cell extracts were electrophoresed through a 10% polyacrylamide gel containing SDS, followed by western blotting with antibodies against FLAG-tag and His-tag. The amount of RNase E did not change after T4 infection.

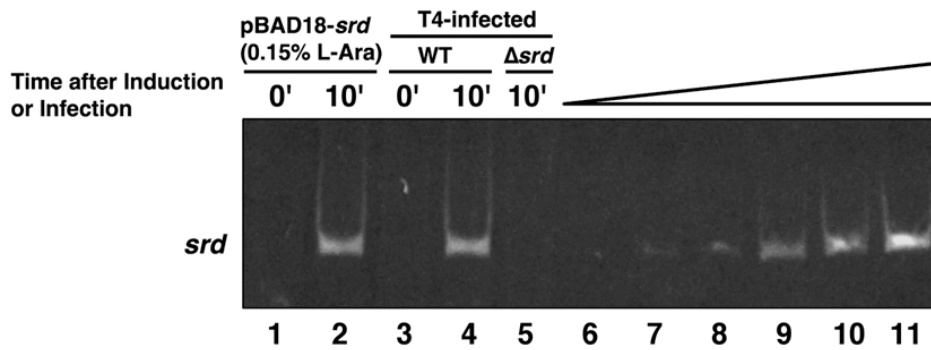


Figure S6 Semi-quantitative RT-PCR analysis to detect *srd* mRNA from T4 genome. Total RNAs from TY0807 cells harboring pBAD18-*srd* at 0 or 10 min after induction of 0.15% arabinose (lanes 1 and 2), or from MH1 cells after infection with wild-type phage (lanes 3 and 4) or Δ *srd* mutant (lane 5) were used for RT-PCR. RT-PCR analyses for *srd* mRNA were performed by incubation at 42° for 50 min with 2 μ g of total RNA, 80 units of ReverTra Ace reverse transcriptase (TOYOBO), 1 mM of dNTPs, and 2.5 pmol of *srd*-RT primer (5'-ACGCGTCGACTTATCCTCGGATAAG) in 10 μ l of reverse transcription buffer. PCR amplification was performed with 0.2 mM dNTPs, 1 μ l of reverse transcription mixture, 10 pmol of sense primer (5'-CGCATAGCAGAAGGCGCTGAAG) and antisense primer (5'-GCGGATATCCTCTTTTCAGTTT), and 1 unit of KOD Dash (TOYOBO) in 25 μ l of PCR Buffer. A thermal cycle of 94° for 30 s, 54° for 15 s, and 72° for 20 s was repeated 15 times. The products were separated through a 5% polyacrylamide gel. Various amounts of pBAD18-*srd* were used as a template to demonstrate a semi-quantitative profile of PCR conditions; lane 6: 0.1 ng; lane 7: 0.5 ng; lane 8: 1 ng; lane 9: 2.5 ng; lane 10: 5 ng; lane 11: 10 ng. The level of *srd* mRNA during T4 infection was almost same as that derived from the plasmid.