

## Periplasmic Domains Define Holin-Antiholin Interactions in T4 Lysis Inhibition

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**Bacteriophage T4 effects host lysis with a holin, T, and an endolysin, E. T and E accumulate in the membrane and cytoplasm, respectively, throughout the period of late gene expression. At an allele-specific time, T triggers to disrupt the membrane, allowing E to enter the periplasm and attack the peptidoglycan. T triggering can be blocked by secondary infections, leading to the state of lysis inhibition (LIN). LIN requires the T4 antiholin, RI, and is sensitive to the addition of energy poisons. T is unusual among holins in having a large C-terminal periplasmic domain. The *rI* gene encodes a polypeptide of 97 residues, of which 72 are predicted to be a periplasmic domain. Here, we show that the periplasmic domain of RI is necessary and sufficient to block T-mediated lysis. Moreover, when overexpressed, the periplasmic domain of T ( $T_{CTD}$ ) was found to abolish LIN in T4 infections and to convert wild-type (wt) T4 plaques from small and fuzzy edged to the classic “r” large, sharp-edged plaque morphology. Although RI could be detected in whole cells, attempts to monitor it during subcellular fractionation were unsuccessful, presumably because RI is a highly unstable protein. However, fusing green fluorescence protein (GFP) to the N terminus of RI created a more stable chimera that could be demonstrated to form complexes with wild-type  $T_{CTD}$ , and also with its LIN-defective T75I variant. These results suggest that the function of the unusual periplasmic domain of T is to transduce environmental information for the real-time control of lysis timing.**

An *Escherichia coli* cell infected at 37°C by a wild-type (wt) T4 phage undergoes lysis at about 25 min and releases ~200 progeny virions. Lysis requires the muralytic activity of the T4 lysozyme, E, one of the best characterized soluble enzymes in terms of its structure, enzymatic mechanism, and thermodynamic stability (26). The precise timing of lysis, however, is not determined by E, which accumulates fully folded and active in the cytoplasm throughout the morphogenesis period. Instead, like all double-stranded DNA phages, the timing of T4 lysis is controlled by its holin, T, an integral membrane protein that suddenly triggers to disrupt the bilayer at an allele-specific time (35, 39). Membrane disruption allows the T4 lysozyme to attack the cell wall, after which the infected cell bursts and releases the progeny virions. T4 *t*, like the  $\lambda$  holin gene *S*, is genetically malleable, in that many missense alleles have been isolated, with lysis times either advanced or delayed relative to the wt allele (13, 28, 31, 35, 37). This malleability is significant, because it is thought that investing lysis timing exclusively in the holin gene allows double-stranded DNA phages to evolve rapidly in response to changed conditions. For example, an environment with reduced host numbers should favor phages with an extended latent period, allowing the intracellular accumulation of more progeny virions before they are released into the host-poor medium (34).

Holin genes have enormous diversity, with more than 50 unrelated gene families having been described (35). However, compared to other known holins, the T4 holin has an unusual topology. All other characterized holins have either three

(class I; e.g., the S105 product of phage  $\lambda$  gene *S*) or two (class II; e.g., the  $S^{2168}$  product of  $S^{21}$ , the holin gene of lambdoid phage 21) transmembrane domains (TMDs) (Fig. 1A). In contrast, T and its orthologs in T4-like phages constitute a single protein family with bitopic topology (Fig. 1A and 2). T, at 218 residues, is substantially larger than other holins (e.g., the  $\lambda$  holin S105 is only 105 residues and the  $S^{2168}$  is only 68 residues). The extra mass comes principally from its large C-terminal periplasmic domain of 163 residues (Fig. 2A).

In addition to its unusual topology and size, the function of the T4 holin is subject to a type of control not seen with the prototypical class I and II holins. Almost 60 years ago, in publications now considered landmarks in the history of molecular genetics, it was reported that T4-infected cells are subject to “lysis inhibition,” or LIN (8, 16). The LIN state, in which the normal lysis timing of the holin is overridden, is established if a T4-infected cell undergoes superinfection by another T4 particle. The LIN state is unstable, requires continued superinfection to be maintained, and can be subverted by addition of energy poisons that collapse the membrane potential (1). In infected cultures at visible optical densities, the lysis of a small fraction of the cells generates sufficient free virions in the medium to establish and maintain LIN throughout the bulk culture, allowing progeny to accumulate to  $>10^3$  virions per cell over a period of hours. On agar lawns, LIN causes T4 to make small, indistinct plaques, and it is easy to isolate T4 mutants defective in LIN by virtue of their large, clearly defined plaque morphology (15). These mutants were called *r* mutants (for “rapid lysis”) and were mapped to multiple T4 loci, including *rI*, *rILAB*, *rIII*, and *rV*, depending on the host used (37). The *r* genetic system was extensively exploited to establish many of the fundamental principles of molecular genetics (7). Ultimately, only two genes, *rI* (3, 27) and *rV* (20,

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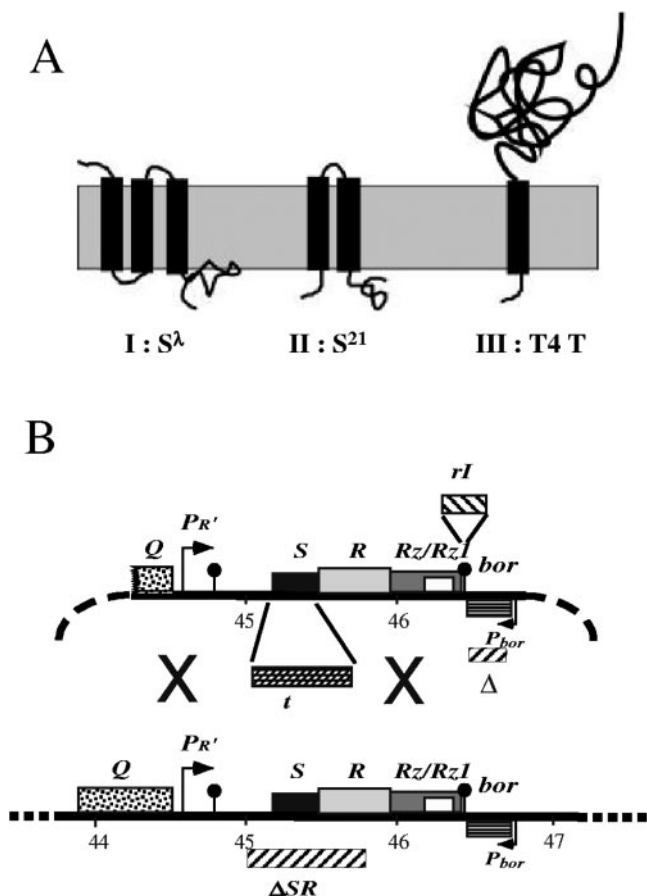


FIG. 1. (A) Topology of class I, II, and III holins (35, 38). (B) Features of plasmid and phage constructs. All plasmids used for inductions are based on the structure of pS105 (14) (upper structure), with the  $\lambda$  late promoter and the promoter-proximal genes of the late transcriptional unit (lower structure), except that in the plasmid pT4T, the *S* gene in the  $\lambda$  SRRzRzI lysis cassette of pER157 (30) is replaced by the phage T4 *t* gene. In plasmid pT4TRI, the *rI* gene is inserted 13 nucleotides downstream of the *Rz* gene of pT4T. "X" designates areas where homologous recombination can occur for generation of  $\lambda t$  recombinants.

21), later shown to be allelic to *t* (10), are required to maintain the wild-type plaque phenotype and to establish LIN with *E. coli* K-12. Nevertheless, despite the central importance of the *r* genetic system in the history of molecular biology, the molecular basis of LIN has remained obscure.

Recently, we have undertaken a molecular analysis of T4 lysis and the LIN phenomenon as part of our study of the mechanisms of phage lysis and its regulation. We reported evidence that RI (Fig. 2B) is an antiholin that specifically binds to and inhibits the T holin. This clearly distinguishes T4 from bacteriophage  $\lambda$ , whose antiholin, S107, is the product of an alternative translational start in its holin gene, *S*, which also encodes S105, the  $\lambda$  holin. Given its near identity with S105 and the fact that holins oligomerize in the process of forming membrane lesions, it is not surprising that S107 dimerizes with S105. The formation of these dimers is responsible for the ability of S107 to prevent the spontaneous triggering of S105. In contrast, RI has no sequence similarity to T (Fig. 2) that

might support homotypic interactions of the type observed in the  $\lambda$  S105/S107 system. Here we report experiments designed to identify the topological determinants of RI and T that lead to the LIN state. The results are discussed in terms of the unique ability of RI to respond to an environmental signal (i.e., a superinfecting T4 phage).

## MATERIALS AND METHODS

**Bacterial strains, bacteriophages, plasmids, and culture growth.** The bacterial strains, bacteriophages, and plasmids used in this work are described in Table 1. T4 phage stocks were prepared as described previously (30). Bacterial cultures were grown in standard LB medium supplemented with ampicillin (100  $\mu$ g/ml), kanamycin (40  $\mu$ g/ml), and chloramphenicol (10  $\mu$ g/ml) when appropriate. Growth and lysis of cultures were monitored by *A*<sub>550</sub> as previously described (30). When indicated, isopropyl  $\beta$ -D-thiogalactoside (IPTG), KCN, or CHCl<sub>3</sub> was added to give final concentrations of 1 mM, 10 mM, or 1%, respectively.

**Standard DNA manipulations, PCR, and DNA sequencing.** Isolation of plasmid DNA, DNA amplification by PCR, DNA transformation, and DNA sequencing were performed as previously described (36). Oligonucleotides were obtained from Integrated DNA Technologies, Coralville, IA, and were used without further purification. The sequences of the oligonucleotides used are listed in Table 2. The Rapid DNA ligation kit from Roche Molecular Biochemicals was used for ligation reactions. All other enzymes were purchased from New England Biolabs, except for *Pfu* polymerase, which was from Stratagene. Automated fluorescent sequencing was performed at the Laboratory for Plant Genome Technology at the Texas Agricultural Experiment Station.

Single-base changes and small insertions were introduced using commercially synthesized primers in conjunction with the QuikChange kit from Stratagene. Larger insertions, replacements, and gene fusions were generated using a modification of the basic QuikChange site-directed mutagenesis protocol. Here, a donor sequence is PCR amplified using primers that have 5' ends that anneal to appropriate sequences in a target plasmid. The first PCR product is then used as the primer for a second PCR using the target plasmid as a template. All subsequent steps are identical to those in the basic QuikChange protocol.

**Construction of plasmids.** pT4T was derived by removing the *aphI* (kanamycin resistance) gene from pER-t (30) and was a gift from L.-N. Wang. It carries a hybrid lysis cassette in which the T4 *t* gene (Fig. 2A, nucleotides [nt] 160204 to 160884 of the T4 genome) replaces the  $\lambda$  *S* gene (nt 45157 to 45465 of the  $\lambda$  genome) in a DNA segment comprising pR', the  $\lambda$  late promoter, the downstream genes SRRzRzI, and a deletion of the *bor* gene (Fig. 1B). This lysis cassette is flanked by unique HindIII and ClaI sites (not shown). The plasmid pT4TRI was constructed by PCR amplification of the lysis cassette from pT4T using the forward and reverse primers HindIIIpR' for and CRzNR1rev. In a separate PCR, the *rI* gene was amplified using the forward and reverse primers CRzNR1for and ClaIR1rev. The *rI* gene in the template used for this reaction had its internal ClaI site destroyed by introduction of the silent mutation G63A by site-directed mutagenesis. Since the primers CRzNR1rev and CRzNR1for are complementary, it was possible to fuse the *rI* gene sequence (nt 59540 to 59177 of the T4 genome) to the 3' end of the hybrid lysis cassette (after the base corresponding to 46437 of the  $\lambda$  genome, beyond the end of the *Rz* gene; Fig. 1B) by using the two PCR products as templates in a splicing by overlapping extension (SOE) reaction (17) using the HindIIIpR' for and ClaIR1rev primers. The product from this reaction was digested with HindIII and ClaI and ligated into the vector backbone produced by digesting pT4T with the same enzymes. The plasmid pT4T<sup>his</sup> was generated by introducing a hexahistidine tag between codons 132 and 133 of the *t* gene (Fig. 2A) in pT4T, using a pair of oligonucleotides, 132-H6G2for/rev, encoding His<sub>6</sub>Gly<sub>2</sub>.

Two plasmids were constructed for overexpression of the His-tagged C-terminal domains of RI (RI<sub>CTD</sub><sup>his</sup>) and T (T<sub>CTD</sub><sup>his</sup>). For the T<sub>CTD</sub><sup>his</sup> plasmid, the DNA fragment encoding T<sub>CTD</sub><sup>his</sup> was PCR amplified from pT4T<sup>his</sup> using the primer pair NdeI70tfor and BamHI1rev. The doubly digested PCR product was inserted into the multiple cloning site of plasmid pET11a to generate the plasmid pET11a-T<sub>CTD</sub><sup>his</sup>. To construct the RI<sub>CTD</sub><sup>his</sup> plasmid, a fragment carrying gene *rI* was produced by cleaving pZE12-RI with EcoRI and XbaI. The plasmid pER-t was digested using the same enzymes to generate the backbone for the plasmid pPRI, and this backbone fragment was ligated to the EcoRI-XbaI fragment carrying *rI* to produce pPRI. The plasmid pPRI<sup>his</sup> was made by inserting hexahistidine tag after codon 97 of the *rI* gene (Fig. 2B) in pPRI, using the primer pair, RIG2H6for/rev, encoding Gly<sub>2</sub>His<sub>6</sub>. The DNA fragment encoding the RI<sub>CTD</sub><sup>his</sup> PCR was amplified from pPRI<sup>his</sup> using 25RINdeIfor and RIhisBamHI1rev with NdeI and BamHI restriction sites at their 5' ends, respec-

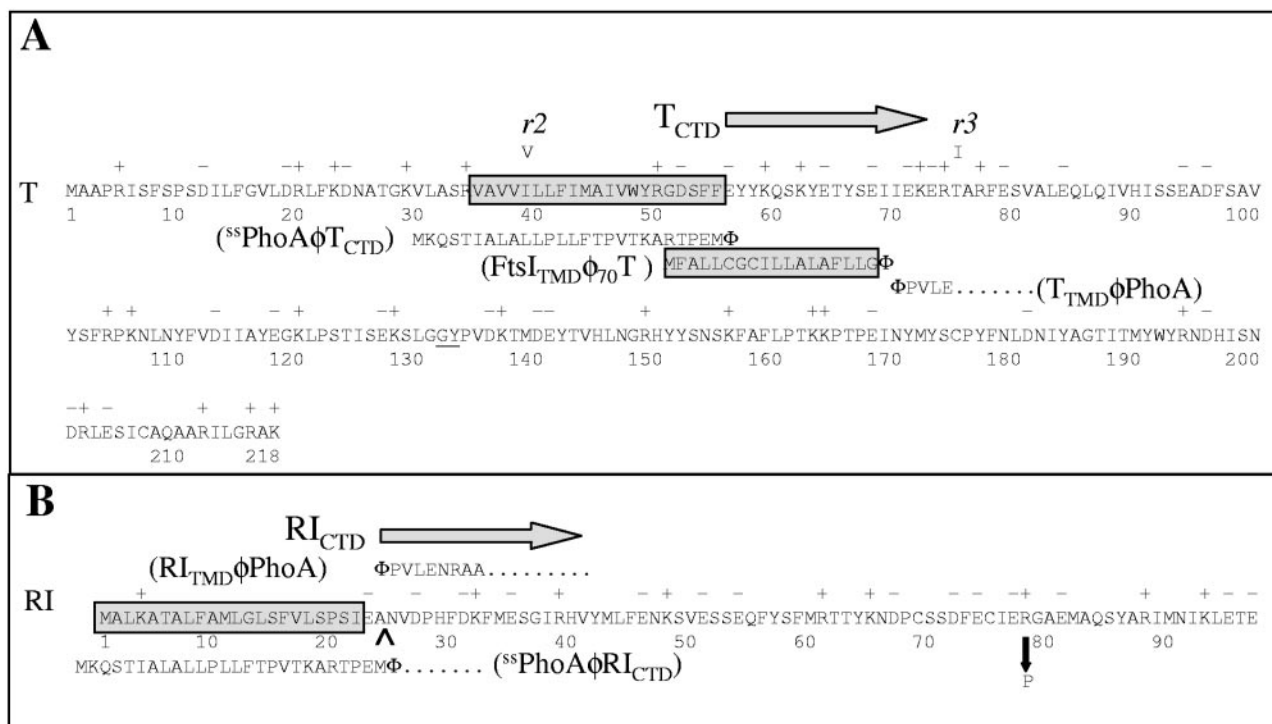


FIG. 2. (A) Amino acid sequence of T and its derivatives. The predicted TMD of T is boxed and shaded. The *rV* mutants *r2*(I39V) and *r3*(T75I) are indicated above the sequence. In T<sup>his</sup>, a hexahistidine tag (HHHHHHGG) was inserted between the residues G132 and Y133 (underlined). For the <sup>ss</sup>PhoAΦT<sub>CTD</sub> construct, the signal sequence of PhoA, shown below the T sequence, was substituted for residues 1 to 55 of T. For the FtsI<sub>TMD</sub>ΦT construct, the transmembrane domain of FtsI, shown in a shaded box below the T sequence, was substituted for residues 2 to 69 of T. For the T<sub>TMD</sub>ΦPhoA construct, residues 1 to 70 of T were substituted for residues 1 to 26 in the full-length primary gene product of *phoA*; the PhoA sequence begins with PVLE (from PhoA residue 27 on, as indicated). (B) Amino acid sequence of RI and its derivatives. The predicted signal sequence of RI is boxed, with the leader peptidase I cleavage site predicted by the SignalP program (<http://www.cbs.dtu.dk/services/SignalP/>) (2) indicated by a carat. In the *c-myc*-tagged version of RI, the *c-myc* tag (QKLISEEDL) was inserted after the terminal glutamate at position 97, while in the GFPΦRI<sup>cmyc</sup> constructs, the entire GFP sequence was inserted after the initial Met residue. For the <sup>ss</sup>PhoAΦRI<sub>CTD</sub> construct, the signal sequence of PhoA, shown below the RI sequence, was substituted for residues 1 to 24 of RI. For the RI<sub>NTD</sub>ΦPhoA construct, the predicted signal sequence of RI replaced residues 1 to 26 in the uncleaved precursor to mature PhoA. The sole known *rI* missense mutation, R78P, is shown by a down arrow.

tively. The doubly digested PCR product was inserted into the multiple cloning site of pET11a to yield pET11a-RI<sub>CTD</sub><sup>his</sup>.

λ<sup>his</sup> was generated by homologous recombination between pT4<sup>his</sup> and the lysis-defective phage λkanΔ(*SR*) (formerly designated as λΔ*SR*) (28) (Fig. 1B). Recombinants were identified by their plaque-forming ability, and the presence of the hybrid lysis cassette was verified by DNA sequencing. Lysogens were prepared by infecting cells with λ<sup>his</sup> and plating at 30°C for survivors on media containing kanamycin.

The T4 *t* and *rI* genes and their derivatives were also expressed from the *lac* promoter of the pZA and pZE plasmids from the family of modular pZ vectors (25). To construct pZA-RI and pZA-RI<sup>cmyc</sup>, the primer pair, Kpn1RIfor and RIAvrIIrev was used to PCR amplify the *rI* gene from pZE-RI or pZE-RI<sup>cmyc</sup>, respectively. After digestion with KpnI and AvrII, these PCR products were used to replace the *luc* gene in pZA32-*luc*. The plasmid pZA-RI<sub>CTD</sub>, carrying a DNA fragment encoding the C-terminal domain (residues 25 to 97; Fig. 2B) of the RI protein, was similarly constructed using the primers 25RIKpnIfor and RIAvrI-Rev. The signal sequence of alkaline phosphatase (PhoA) was fused to the RI fragment in pZA-RI<sub>CTD</sub> by the modified site-directed mutagenesis procedure described above. In the first PCR, the PhoA signal sequence was amplified using the ss25RIfor and ss25RIrev primers. The second PCR used pZA-RI<sub>CTD</sub> as the template yielding pZA-<sup>ss</sup>PhoAΦRI<sub>CTD</sub>. The identical reactions were used to generate pZA-RI<sub>CTD</sub><sup>cmyc</sup> and pZA-<sup>ss</sup>PhoAΦRI<sub>CTD</sub><sup>cmyc</sup> from pZA-RI<sup>cmyc</sup>. A similar strategy was used to generate the complementary series of plasmids pZA-T, pZA-T<sub>CTD</sub>, and pZA-<sup>ss</sup>PhoAΦT<sub>CTD</sub>, using the primer pairs KpnIfor/AvrIIrev, 56tKpnIfor/AvrIIrev, and ss25RIfor/56tss rev, respectively. Here, the T<sub>CTD</sub> consists of residues 56 to 218 of T (Fig. 2A).

The sequence encoding residues 27 to 471 of the *phoA* gene was PCR ampli-

fied using the forward and reverse primers KpnIphoAfor and AvrIIphoArev, with KpnI and AvrII restriction sites at their 5' ends, respectively. The doubly digested PCR product was used to replace the *luc* gene in pZA32-*luc* to yield pZA-PhoA. To fuse the N-terminal domain (residues 1 to 24, RI<sub>NTD</sub>) of RI to the mature form of PhoA, the DNA encoding the RI<sub>NTD</sub> was PCR amplified using the primers RI<sub>NTD</sub>phoAfor and RI<sub>NTD</sub>phoArev. The PCR product was then used to conduct a modified site-directed mutagenesis reaction using pZA-PhoA as the template to generate pZA-RI<sub>NTD</sub>ΦPhoA. The plasmid pZA-T<sub>TMD</sub>ΦPhoA, in which residues 1 to 70 of T are fused to the mature sequence of PhoA, was constructed in a similar fashion using the primers T4T<sub>TMD</sub>phoAfor and T4T<sub>TMD</sub>phoArev.

The *ftsI<sub>tmd</sub>φt* chimera, encoding a protein with the TMD of FtsI (Fig. 2A) replacing the TMD of T, was constructed in two steps. First, a DNA fragment encoding residues 70 to 218 of T was PCR amplified using the forward and reverse primers AvrII<sub>70</sub>rev and KpnI<sub>70</sub>for, with AvrII and KpnI restriction sites at their 5' ends, respectively. The doubly digested PCR product was used to replace the *luc* gene in pZA32-*luc*, generating the intermediate plasmid, pZA-T<sub>70-218</sub>. Then, a DNA fragment encoding a methionine codon followed by the transmembrane segment of FtsI (residues 24 to 40) was PCR amplified using the forward and reverse primers FtsITM<sub>70</sub>for and FtsITM<sub>70</sub>rev. The PCR product was then used to conduct a modified site-directed mutagenesis reaction by using pZA-T<sub>70-218</sub> as the template to generate pZA-FtsI<sub>TMD</sub>ΦT.

Green fluorescent protein (GFP) was fused to *c-myc*-tagged RI (Fig. 2B) by another SOE reaction. First, a DNA fragment encoding GFP was PCR amplified from pDS439 (33) using the primers KpnI<sub>gfp</sub>for and N-RIC-gfp<sub>rev</sub>. Separately, a DNA fragment encoding residues 2 to 106 of the *c-myc*-tagged RI protein was PCR amplified from pZE-RI<sup>cmyc</sup> using the primers C-gfpN-RIfor and RI-

TABLE 1. Phages, strains, and plasmids used in this work

Phage, strain, or plasmid	Genotype/features	Source or reference
<b>Phages</b>		
T4 wt	T4D	I. Molineux
T4 $rl$	<i>rl48</i> , single-base deletion at position 195; N66-E97 replaced with MTRALLILNV	D. Hall
$\lambda$ kan $\Delta$ (SR)	$\lambda$ b <sub>515</sub> b <sub>519</sub> att::Tn903 <i>cI</i> <sub>857</sub> <i>nin5</i> $\Delta$ (SR)	28
$\lambda$ kan <i>t</i> <sup>his</sup>	$\lambda$ b <sub>515</sub> b <sub>519</sub> att::Tn903 <i>cI</i> <sub>857</sub> <i>nin5</i> $\Delta$ S:: <i>t</i> <sup>his</sup>	This study
<b>Strains</b>		
CQ21	<i>E. coli</i> K-12 <i>ara leu lacI</i> <sup>q1</sup> <i>purE gal his argG rpsL xyl mtl ilv</i>	28
CQ21 $\lambda$ kan $\Delta$ (SR)	Lysogen carrying $\lambda$ kan $\Delta$ (SR) prophage	28
CQ21[ $\lambda$ kan $\Delta$ (SR)] <i>recA srl</i> ::Tn10	Lysogen carrying $\lambda$ kan $\Delta$ (SR) prophage	This study
CQ21( $\lambda$ kan <i>t</i> <sup>his</sup> )	Lysogen carrying $\lambda$ kan <i>t</i> <sup>his</sup> prophage	This study
MG1655	F <sup>-</sup> <i>ilvG rfb50 rph1</i>	<i>E. coli</i> Genetic Stock Center
MDS12	MG1655 with 12 deletions, totalling 376,180 nt, including cryptic prophages	19
MDS12 <i>tonA</i> ::Tn10 <i>lacI</i> <sup>q1</sup>		This study
XL-1Blue	<i>E. coli</i> K-12 <i>recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F <sup>+</sup> <i>proAB lacZ</i> <sub><math>\Delta</math>M15</sub> ::Tn10]	Stratagene
BL21(DE3)	F <sup>-</sup> <i>ompT hsdS<sub>B</sub></i> ( <i>r<sub>B</sub></i> <sup>-</sup> <i>m<sub>B</sub></i> <sup>-</sup> ) <i>gal dcm</i> (DE3)	Novagen
BL21(DE3) <i>tonA</i> ::Tn5 <i>slyD</i> ::Tet <sup>r</sup>		This study
<b>Plasmids</b>		
pT4T	pBR322 derivative carrying late promoter and lysis cassette of $\lambda$ with <i>S</i> gene replaced by T4 <i>t</i>	I. N. Wang
pT4T <sup>his</sup>	pT4T with HHHHHHGG inserted between residues 132 and 133 of T	This study
pT4TRI	pT4T with <i>rl</i> gene inserted distal to <i>Rz1</i> gene	This study
pZA32-luc	p15A origin, P <sub>LacO-1</sub> promoter, Cam <sup>r</sup>	25
pZA-RI	pZA32 $\Delta$ <i>luc</i> :: <i>rl</i>	This study
pZA-RI <sub>CTD</sub>	pZA32 $\Delta$ <i>luc</i> :: <i>rl</i> <sub>CTD</sub> , codons 25 to 97 of <i>rl</i>	This study
pZA-PhoA	pZA32 $\Delta$ <i>luc</i> :: <i>phoA</i> , codons 27–471 of <i>phoA</i> , encoding mature PhoA	This study
pZA- <sup>ss</sup> PhoA $\Phi$ RI <sub>CTD</sub>	Codons 1–24 of <i>rl</i> in pZA-RI replaced by codons 1–26 of <i>phoA</i> , encoding signal sequence	This study
pZA- <sup>ss</sup> PhoA $\Phi$ RI <sub>CTD</sub> <sup>cmyc</sup>	pZA- <sup>ss</sup> PhoA $\Phi$ RI <sub>CTD</sub> with 10 codons encoding QKLISEEDL inserted after codon 97 of <i>rl</i>	This study
pZA-RI <sub>TMD</sub> $\Phi$ PhoA	First 24 codons of <i>rl</i> inserted in front of codon 27 of <i>phoA</i> in pZA-PhoA	This study
pZA-T	pZA32 $\Delta$ <i>luc</i> :: <i>t</i>	This study
pZA-T <sub>CTD</sub>	pZA32 $\Delta$ <i>luc</i> :: <i>t</i> <sub>CTD</sub> , codons 56–218 of <i>t</i>	This study
pZA- <sup>ss</sup> PhoA $\Phi$ T <sub>CTD</sub>	Codons 1–55 of <i>t</i> in pZA-T replaced by codons 1–26 codons of <i>phoA</i> , encoding signal sequence	This study
pZA- <sup>ss</sup> PhoA $\Phi$ T <sub>CTD</sub> T75I	pZA- <sup>ss</sup> PhoA $\Phi$ T <sub>CTD</sub> carrying T75I <i>rV</i> mutation	This study
pZA-T <sub>TMD</sub> $\Phi$ PhoA	First 70 codons of <i>t</i> inserted before codon 27 of <i>phoA</i> in pZA-PhoA	This study
pZA-T <sup>70-218</sup>	pZA32 $\Delta$ <i>luc</i> :: <i>t</i> <sup>70-218</sup> , codons 70–218 of <i>t</i>	This study
pZA-FtsI <sub>TMD</sub> $\Phi$ 70T	Codons 24 to 40 of <i>ftsI</i> , encoding TMD, inserted before codon 70 of <i>t</i> in pZA-T <sup>70-218</sup>	This study
pZE12-luc	ColE origin, P <sub>LacO-1</sub> promoter, Amp <sup>r</sup>	25
pZE12-RI	pZE12 $\Delta$ <i>luc</i> :: <i>rl</i>	This study
pZE12-RI <sup>cmyc</sup>	pZE12 $\Delta$ <i>luc</i> :: <i>rl</i> <sup>cmyc</sup> with codons encoding QKLISEEDL inserted after codon 97 of <i>rl</i>	This study
pZE12-GFP $\Phi$ RI <sup>cmyc</sup>	DNA fragment encoding GFP inserted between codons 1 and 2 of <i>rl</i> <sup>cmyc</sup> in pZE12-luc	This study
pDS439	pBR322 origin, P <sub>araBAD</sub> promoter, Amp <sup>r</sup> , carrying <i>gfp</i> <sup>mut2</sup>	33
pET11a-T <sub>CTD</sub> <sup>his</sup>	pBR322 origin, T7 promoter, Amp <sup>r</sup> , carrying DNA fragment encoding Met residue, then residues 70–218 of T with HHHHHHGG insertion between residues 132 and 133	This study
pER-t	Carrying lysis cassette of $\lambda$ except <i>S</i> is replaced by T4 <i>t</i> gene, Amp <sup>r</sup> , Kan <sup>r</sup>	30
pPRI	pBR322 origin, $\lambda$ P <sub>R'</sub> promoter, Amp <sup>r</sup> , carrying DNA fragment encoding RI	This study
pPRI <sup>his</sup>	pPRI with 8 codons encoding GGHHHHHH inserted after codon 97 of <i>rl</i>	This study
pET11a-RI <sub>CTD</sub> <sup>his</sup>	pBR322 origin, T7 promoter, Amp <sup>r</sup> , carrying DNA fragment carrying Met codon, then codons 25–97 of RI, followed by codons for sequence GGHHHHHH	This study

Xbarev. The PCR products from the two reactions were combined and amplified using the primers KpnI<sub>gfp</sub> and RI-Xbarev. The fusion product was digested with KpnI and XbaI and ligated into pZE12 digested with the same enzymes, yielding pZE-GFP $\Phi$ RI<sup>cmyc</sup>.

**Subcellular fractionation.** To prepare total membrane and soluble fractions, cell pellets from 150-ml cultures were resuspended in 1 ml of French press buffer (100 mM Na<sub>2</sub>HPO<sub>4</sub>, 100 mM KCl, 5 mM EDTA, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 25 mM MgCl<sub>2</sub>, 50  $\mu$ g/ml DNase, 50  $\mu$ g/ml RNase) and 2.5  $\mu$ l

of protease inhibitor cocktail [4-(2-aminoethyl)benzenesulfonyl fluoride, bestatin, pepstatin, E-64, and phosphoramidon; Sigma]. The cells were disrupted by passage through a French pressure cell (Spectronic Instruments, Rochester, N.Y.) at 16,000 lb/in<sup>2</sup> (1 lb/in<sup>2</sup> = 6.89 kPa). The unbroken cells were removed by centrifugation in a Damon-IEC Spinette clinical centrifuge at 1,000  $\times$  *g* for 10 min. The membrane and soluble fractions were separated by centrifugation at 100,000  $\times$  *g* for 60 min at 4°C. To identify periplasmic proteins, cells from 30-ml cultures were collected by centrifugation and the pellets were resuspended in 250



## RESULTS

**Domain analysis of the RI antiholin.** Paddison et al. (27) used primary structure analysis algorithms to predict that RI has an N-terminal secretory signal and was thus a secreted periplasmic protein. Although RI has been visualized as a cross-linked complex with T, it had previously escaped detection as an independent polypeptide, which presumably was due to proteolytic lability (29). Thus, whether this N-terminal sequence serves as a signal anchor, leaving its periplasmic C-terminal domain (RI<sub>CTD</sub>) tethered to the cytoplasmic membrane, or a cleavable signal allowing release of the RI<sub>CTD</sub> into the periplasm was unclear. Sequence analysis of the classical *rI* alleles of Doermann (9) revealed that most were frameshifts distal to the predicted secretory signal. Moreover, the single missense allele (R78P; listed as R78G by Paddison et al. [27]) (Fig. 2B), also mapped there, implicating RI<sub>CTD</sub> as critical to the antiholin function of RI.

To resolve RI into topological components, we constructed two chimeric genes: one, <sup>ss</sup>*phoA*Φ*rI*<sub>CTD</sub>, with RI<sub>CTD</sub> fused to the signal sequence of alkaline phosphatase (PhoA); the other, *rI*<sub>NTD</sub>Φ*phoA*, with the N-terminal domain of RI (RI<sub>NTD</sub>) fused to the periplasmic domain of PhoA (Fig. 2B). Using antibodies raised against RI<sub>CTD</sub>, the <sup>ss</sup>PhoAΦRI<sub>CTD</sub><sup>cmyc</sup> protein could be detected in whole cells (Fig. 3A). However, efforts to localize it using conventional subcellular fractionation were unsuccessful, again presumably due to rapid proteolysis after cell disruption. To provide evidence that <sup>ss</sup>PhoAΦRI<sub>CTD</sub><sup>cmyc</sup> was present in the periplasm, the <sup>ss</sup>*phoA*Φ*rI*<sub>CTD</sub> gene was expressed in cells grown in the presence or absence of azide to inhibit SecA. A slower-migrating species accumulated in the presence of azide, indicating that, when SecA is not inhibited, <sup>ss</sup>PhoAΦRI<sub>CTD</sub><sup>cmyc</sup> is processed and localized to the periplasm (Fig. 3A). In contrast, the product of the *rI*<sub>NTD</sub>Φ*phoA* construct was stable, unprocessed, and was found in both the membrane and soluble fractions (Fig. 3B). The dual localization of the unprocessed RI<sub>NTD</sub>ΦPhoA protein will be considered elsewhere (T. A. T. Tran., D. K. Struck, and R. Young, manuscript in preparation). We next compared the ability of these chimeras to support LIN with that of wt RI. While the *rI*<sub>NTD</sub>Φ*phoA* fusion had no biological function, the <sup>ss</sup>*phoA*Φ*rI*<sub>CTD</sub> chimera blocked *t*-mediated lysis, as assessed by monitoring the turbidity of the induced culture (Fig. 4A). Moreover, in addition to preventing the loss of optical density, both *rI* and <sup>ss</sup>*phoA*Φ*rI*<sub>CTD</sub> allowed the extended intracellular accumulation of virions and suppressed their release to the medium (Fig. 4B). Finally, the LIN state supported by both *rI* and <sup>ss</sup>*phoA*Φ*rI*<sub>CTD</sub> could be subverted by the addition of energy poisons (Fig. 4A). We conclude that the periplasmic domain of RI is necessary and sufficient for authentic LIN. Indeed, the <sup>ss</sup>*phoA*Φ*rI*<sub>CTD</sub> allele is more effective than the parental *rI* gene, as judged by the stability of the LIN phenotype (Fig. 4A).

**RI-dependent LIN requires binding to the C-terminal domain of T.** The unusually large C-terminal periplasmic domain of T (T<sub>CTD</sub>) is a feature that distinguishes it from the numerous class I and class II holins (35). To determine whether this domain is the target for RI<sub>CTD</sub>, we constructed an allele of *t* in which the sequences encoding the predicted cytoplasmic and transmembrane domains were replaced by the segment of *phoA* encoding its secretory signal sequence (<sup>ss</sup>*phoA*Φ*t*<sub>CTD</sub>; Fig.

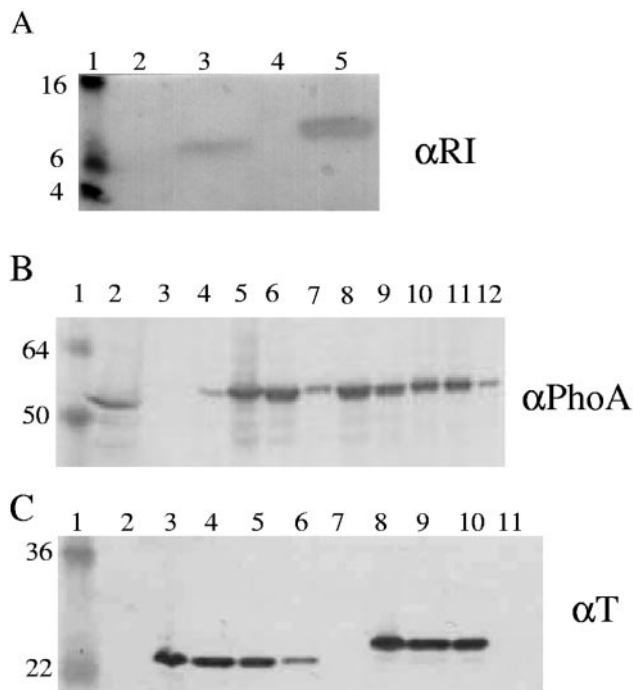


FIG. 3. Localization of T and RI chimeras. Subcellular fractions were prepared and analyzed by SDS-PAGE and Western blotting as described in Materials and Methods. (A) Cells carrying pZA-<sup>ss</sup>PhoAΦRI<sub>CTD</sub><sup>cmyc</sup> were grown in the absence (lanes 2 and 3) or the presence (lanes 4 and 5) of 1 mM azide for 10 min in advance of induction. Cells from these cultures were collected by TCA precipitation and centrifugation, resuspended in SDS-PAGE sample buffer, and subjected to SDS-PAGE and Western blotting using anti-RI antisera as the primary antibody. Lane 1, molecular mass standards; lanes 2 and 4, samples from uninduced cultures; lanes 3 and 5, samples from induced cultures. (B) Cells carrying the pZA-RI<sub>NTD</sub>ΦPhoA were induced, fractionated, and analyzed by SDS-PAGE and Western blotting using anti-PhoA as the primary antibody. Lane 1, molecular mass standards; lane 2, mature form of PhoA; lane 3, blank; lane 4, cells from an uninduced culture; lane 5, cells from an induced culture; lane 6, total cell lysate; lane 7, 1,000 × g pellet; lane 8, 1,000 × g supernatant; lane 9, 100,000 × g supernatant (soluble fraction); lane 10, 100,000 × g pellet (membrane fraction); lane 11, detergent-extractable (1% NP40) membrane fraction; lane 12, detergent-insoluble fraction. (C) Cells carrying pZA-<sup>ss</sup>PhoAΦT<sub>CTD</sub> were grown in the absence (lanes 2 to 6) or the presence (lanes 7 to 11) of 1 mM azide for 10 min in advance of induction, harvested, fractionated, and analyzed by SDS-PAGE and Western blotting using anti-T antisera as the primary antibody. Lane 1, molecular mass standards; lanes 2 and 7, uninduced cells; lanes 3 and 8, induced cells; lanes 4 and 9, cells after spheroplasting; lanes 5 and 10, spheroplasts; lanes 6 and 11, periplasm.

2A). Like the RI<sub>CTD</sub>, the T<sub>CTD</sub> was also efficiently secreted by the PhoA signal sequence (Fig. 3C). This allele was lytically incompetent but exerted a weak dominant-negative phenotype in that it caused a short delay in T-mediated lysis (Fig. 4C, inset), suggesting that homotypic interactions in the T<sub>CTD</sub> are involved in the lytic function of T. The biological function of this chimera was assessed in a system in which *t* and *rI* are both expressed from the λ late promoter, shown in previous work to support physiologically meaningful lysis timing with the λ lysis cassette (13). The results clearly showed that supplying periplasmic T<sub>CTD</sub> partially blocked the imposition of LIN (Fig. 4C). More dramatic results were obtained when cells produc-

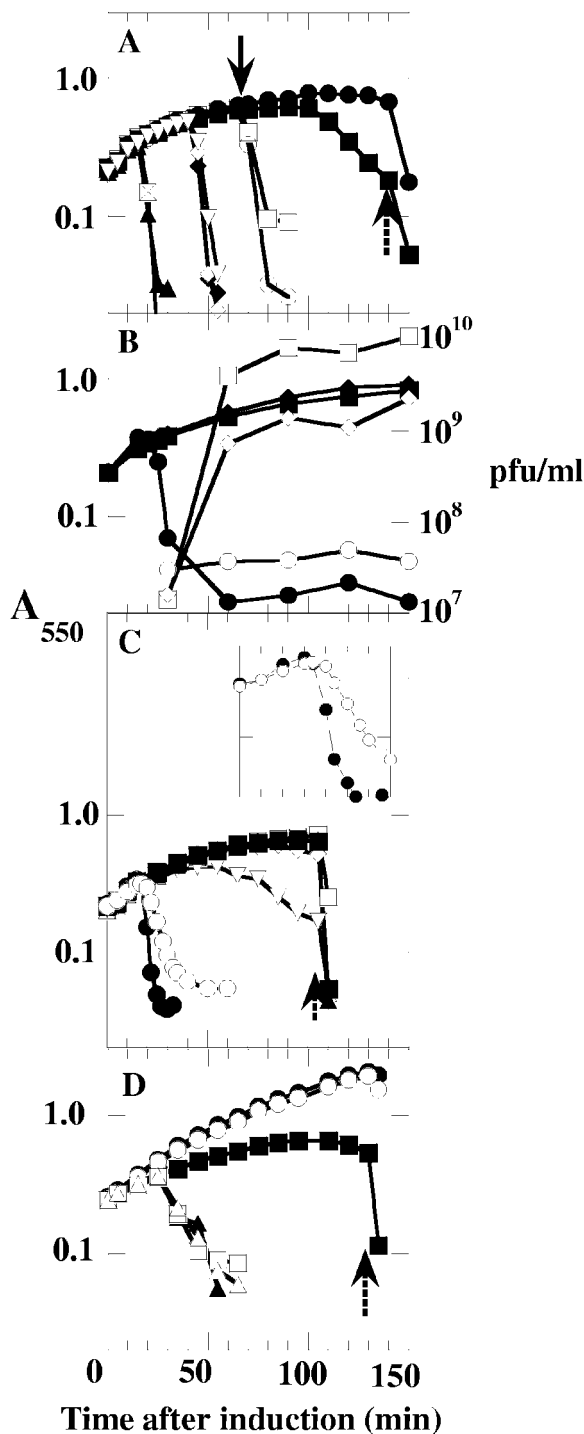


FIG. 4. The C-terminal domains of T and RI are the determinants of LIN. (A)  $^{ss}PhoA\Phi T_{CTD}$  is necessary and sufficient for LIN. CQ21( $\lambda kan\Delta(SR)$ ) cells carrying the indicated plasmids were induced at time zero, and culture turbidity was monitored as a function of time:  $\boxtimes$ , pT4T;  $\blacksquare$ ,  $\square$ , pT4T and pZA-RI;  $\circ$ ,  $\bullet$ , pT4T and pZA- $^{ss}PhoA\Phi RI_{CTD}$ ;  $\blacktriangle$ , pT4T and pZA-RI $_{NTD}\Phi PhoA$ ;  $\blacklozenge$ , pS105;  $\diamond$ , pS105 and pZA-RI;  $\nabla$ , pS105 and pZA- $^{ss}PhoA\Phi RI_{CTD}$ . To demonstrate premature triggering, KCN was added to two cultures (pT4T and pZA-RI,  $\square$ ; and pT4T and pZA- $^{ss}PhoA\Phi RI_{CTD}$ ,  $\circ$ ) at the time indicated by the solid arrow. (B) Phage accumulation during  $^{ss}PhoA\Phi RI_{CTD}$ -mediated LIN. CQ21( $\lambda$ - $t^{his}$ ) carrying the indicated plasmids was induced at time zero, and culture turbidity (solid sym-

ing the  $^{ss}PhoA\Phi T_{CTD}$  were infected with T4 phage; LIN was completely subverted by the presence of the  $T_{CTD}$  (Fig. 4D). In a control infection, induction of  $^{ss}phoA\phi t_{CTD}$  had no effect on the lysis kinetics of T4rI (Fig. 4D). When T4 phage were plated on bacteria secreting  $T_{CTD}$  to the periplasm, wt T4 (T4D) generated large, distinct plaques which were nearly identical to the plaques produced by rI mutants (Fig. 5). We conclude that interactions between the periplasmic domain of RI and the periplasmic domain of T are required for LIN.

**T and RI form a complex.** To provide further evidence that the T and RI proteins interact, we performed coimmunoprecipitation experiments using lysates prepared from cells expressing  $^{ss}PhoA\Phi T_{CTD}$  and a GFP $\Phi RI^{cmyc}$  chimera. The GFP $\Phi RI^{cmyc}$  chimera was used since it was readily visualized by immunoblotting, presumably because of decreased lability compared to either RI or  $^{ss}PhoA\Phi RI_{CTD}$ , both of which were undetectable in immunoprecipitations. Using either anti-T or anti-GFP as the first antibody, the two proteins were found to coprecipitate (Fig. 6A). These complexes were also formed when detergent-solubilized extracts prepared from cells expressing T or GFP $\Phi RI^{cmyc}$  separately were mixed and then subjected to immunoprecipitation (not shown). Identical results were obtained when the T75I mutation found in a *t* allele known to be insensitive to RI-mediated LIN was introduced into the  $^{ss}PhoA\Phi T_{CTD}$  protein (Fig. 6B). Since the R78P allele of rI is defective for LIN, we attempted to test the effect of this mutation on the ability of RI to form complexes with T. Unfortunately, the product of the R78P allele of *gfp $\phi$ rI $^{cmyc}$*  does not accumulate in whole cells to levels detectable by Western blotting.

Interestingly, the ratio of unprocessed  $^{ss}PhoA\Phi T_{CTD}$  to mature periplasmic  $T_{CTD}$  was reproducibly higher when GFP $\Phi RI^{cmyc}$  was present (Fig. 6A and B, lanes 5 to 7). This suggests that the binding of RI to the periplasmic domain can occur while  $T_{CTD}$  is nascent and that this binding interferes with leader peptidase cleavage of the signal sequence.

DISCUSSION

Infections of T4 and the other T-even phages are the only examples where lysis timing has been demonstrated to be directly affected by the environmental conditions surrounding the infected cell. The classic LIN phenotype can be thought of as a “no-quorum” signal, in the sense that an effector molecule

(bols) and phage accumulation (open symbols) were monitored as a function of time.  $\circ$ ,  $\bullet$ , no plasmid;  $\diamond$ ,  $\blacklozenge$ , pZA-RI;  $\square$ ,  $\blacksquare$ , pZA- $^{ss}PhoA\Phi RI_{CTD}$ . (C) Periplasmic  $T_{CTD}$  interferes with LIN. CQ21( $\lambda kan\Delta(SR)$ ) cells carrying the indicated plasmids were induced at time zero, and culture turbidity was monitored as a function of time.  $\bullet$ , pT4T;  $\circ$ , pT4T and pZA- $^{ss}PhoA\Phi T_{CTD}$ ;  $\square$ , pZA- $^{ss}PhoA\Phi T_{CTD}$ ;  $\triangle$ , pT4TRI;  $\nabla$ , pT4TRI and pZA- $^{ss}PhoA\Phi T_{CTD}$ ;  $\diamond$ , pT4TRI and pZA- $T_{CTD}$ ;  $\blacktriangle$ , pT4TRI and pZA- $T_{CTD}$ ;  $\blacklozenge$ , pT4TRI and pZA- $T_{TMD}\Phi PhoA$ . (Inset) Detail for growth of cells carrying pT4T alone ( $\bullet$ ) and pT4T and pZA- $^{ss}PhoA\Phi T_{CTD}$  ( $\circ$ ). (D) Periplasmic  $T_{CTD}$  blocks LIN during T4 phage infections. CQ21 cells carrying either pZA32-luc (solid symbols) or pZA- $^{ss}PhoA\Phi T_{CTD}$  (open symbols) were induced at time zero and were grown without infection ( $\circ$ ,  $\bullet$ ) or infected at a multiplicity of infection of 10 with either T4D ( $\square$ ,  $\blacksquare$ ) or T4rI ( $\triangle$ ,  $\blacktriangle$ ). In panels A, C, and D,  $CHCl_3$  was added at the time indicated by the arrow.

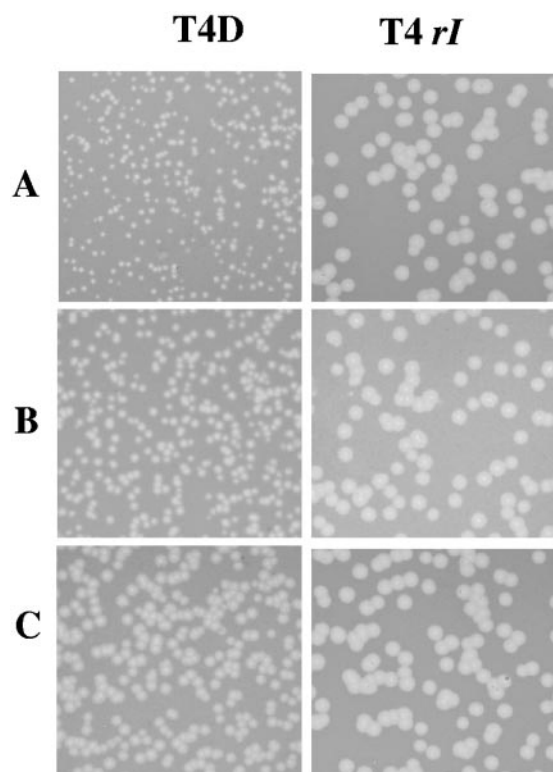


FIG. 5. Periplasmic  $T_{CTD}$  causes T4 to form *r*-type plaques. Lawns of cells of MDS12 *tonA::Tn10 lacI<sup>q1</sup>* (A), MDS12 *tonA::Tn10 lacI<sup>q1</sup>* harboring uninduced pZA- $ssPhoA\Phi T_{CTD}$  (B), or MDS12 *tonA::Tn10 lacI<sup>q1</sup>* harboring induced  $ssPhoA\Phi T_{CTD}$  (C) were infected with either T4D or T4*rI* phage.

(i.e., a superinfecting T4 phage) indicating the lack of available hosts binds to an infected cell and initiates an as yet unknown signal transduction pathway leading to activation of an effector, RI. The simplest interpretation of our data is that activated RI directly binds to and inhibits the target molecule, T, so that lysis is blocked. Both RI and T have hydrophobic domains near their N termini, but the results presented here demonstrate conclusively that the interactions essential for the transmission of the signal are entirely between the periplasmic domains of the two proteins. Moreover, we have shown that RI and T are present in a complex that can be recovered by immunoprecipitation without prior covalent cross-linking. Unlike class I and II holins, which consist of two or three transmembrane domains, short interconnecting loops, and a 10- to 25-residue C-terminal cytoplasmic domain, T and its orthologs in T4-like phage have a single transmembrane domain and a relatively large C-terminal periplasmic domain (31). Our findings suggest that this periplasmic domain has evolved to serve as a receptor for the LIN signal provided by “activated” RI. This idea is consistent with the observation that large deletions within the  $T_{CTD}$  can be tolerated without entirely abolishing holin function (31). In addition, the coimmunoprecipitation experiments also suggest that RI binds to nascent T, since the presence of GFP $\Phi RI^{cmyc}$  appears to reduce the efficiency of the maturation of  $ssPhoA\Phi T_{CTD}$  (Fig. 6A, lanes 5 to 7). This is consistent with the finding that in T4 infections, T4 superinfection can confer RI-mediated LIN on a preexisting pool of wt

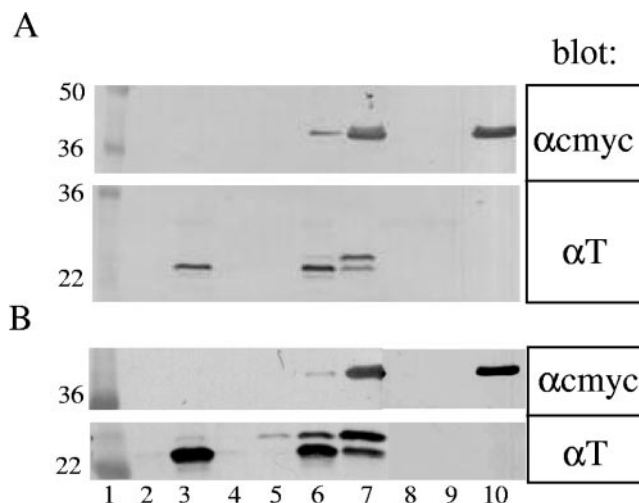


FIG. 6. T and RI form a complex. (A) Immunoprecipitations were performed with samples containing either  $ssPhoA\Phi T_{CTD}$  (lanes 2 to 4), both  $ssPhoA\Phi T_{CTD}$  and GFP $\Phi RI^{cmyc}$  (lanes 5 to 7), or GFP $\Phi RI^{cmyc}$  only (lanes 8 to 10), prepared from induced cells carrying either of the plasmids pZA- $ssPhoA\Phi T_{CTD}$  or pZE-GFP $\Phi RI^{cmyc}$  or both. Primary antibodies for immunoprecipitations: lanes 2, 5, and 8, rabbit preimmune serum; lanes 3, 6, and 9, anti-T rabbit antibody; lanes 4, 7, and 10, anti-GFP monoclonal antibody. Samples were analyzed by SDS-PAGE and immunoblotted with either anti-T or polyclonal anti-*c-myc*, as indicated to the right. (B) Same as panel A, except that the *rV* variant  $ssPhoA\Phi T_{CTD}^{T751}$ , produced from the plasmid pZA- $ssPhoA\Phi T_{CTD}^{T751}$ , was used. The molecular mass appears in lane 1 for each blot.

LIN-sensitive T molecules if the incoming phage has a wt, LIN-sensitive *t* allele, but not if it has an *rV*, LIN-insensitive *t* allele (30). The interference with signal sequence processing also suggests that conformational changes derived from binding of RI to the  $T_{CTD}$  can be transmitted to the membrane, which is consistent with the fact that one of the classic *rV*, LIN-defective mutations is a subtle missense change, I39V, in the hole-forming TMD of T.

The classic LIN phenotype appears to involve a direct interaction between the periplasmic domains of RI and T and can be subverted by the collapse of the membrane potential by energy poisons. Thus, at a superficial level, the antiholin function of RI resembles those of S107 and S<sup>21</sup>71, the antiholins of  $\lambda$  and lambdoid phage 21, respectively. The latter two proteins are identical to their cognate holins except for a short N-terminal extension. In both cases, antiholin-holin interactions are thought to prevent quaternary rearrangements that are required to convert holin oligomers into “holes.” We suggest that in the energized membrane, the single TMD of T is unable to oligomerize into a functional “hole” in the absence of interactions between  $T_{CTD}$ s in the periplasm. The antiholin activity of RI would then be due to its ability to bind to the  $T_{CTD}$  preventing these interactions. Mutations that alter the periplasmic interactions between  $T_{CTD}$  molecules and between  $T_{CTD}$  and  $RI_{CTD}$  would allow for the genetic malleability of lysis timing for T4. For  $\lambda$  bacteriophages (4, 11, 12, 28) and the lambdoid phage 21 (T. Park, D. K. Struck, and R. Young, submitted), collapse of the membrane potential allows premature triggering of the holin by causing topological changes in



the antiholin that lead to its inactivation. Moreover, in these cases, representing canonical class I and II holins, respectively, these topological changes in the antiholin effectively convert it into the functional equivalent of its cognate holin. It seems unlikely that T and RI have the same relationship as is seen with the antiholin/holin pairs of phage  $\lambda$  and 21 for several reasons. First, the homotypic interactions that presumably characterize the S105/S107 and S<sup>2171</sup>/S<sup>2168</sup> systems are not possible since T and RI do not share amino acid sequence homology. Second, RI<sub>NTD</sub> is not essential for its antiholin activity, indicating that RI does not directly interact with the hole-forming TMD of the T holin. Finally, in contrast to S107 or S<sup>2171</sup>, RI appears to be a labile protein whose antiholin activity is only realized physiologically under conditions of superinfection.

While the mechanism of action of RI may be fundamentally different from that of S107 or S<sup>2171</sup>, a feature common to the antiholin function of all three proteins is its abrogation by collapse of the membrane potential. Although the reason for this behavior is not obvious, we propose that it endows bacteriophage lysis systems with a "sentinel" function. Here, the injection of a heterologous phage DNA into a previously infected cell is detected by the resident holin as a transitory depolarization of the membrane, associated with the channel formed in the bilayer through which the DNA passes. The resident holin is thus triggered prematurely, aborting the new infection and allowing release of progeny from the initial infection.

The results presented here indicate that the large periplasmic domain of the T4 holin is fundamentally involved in real-time regulation by RI. In the T4 infection cycle, there is evidence that *rI* is transcribed from both early and late promoters (27). Moreover, the RI-dependent LIN phenotype is imposed by superinfections at 3 min after infection and beyond, before the first molecule of T, as a late gene product, is made. Given the temporal relationship between the expression of the *rI* and *t* genes, why does RI not inhibit T-mediated lysis in the absence of superinfection? The answer to this question may lie with the stability of RI. LIN is a transient phenomenon which requires continual reinfection to significantly prolong the latent period of the initial T4 infection (1). This, in itself, suggests that the effector molecule that transmits the LIN signal to the T protein is unstable. In fact, we can detect RI in whole cells collected by trichloroacetic acid (TCA) precipitation, but not if cells are fractionated, which suggests that it is extremely labile. This leads to the parsimonious model that LIN is imposed only if RI reaches a certain level, which can be attained either by virtue of a stabilization signal provided by superinfection or by over-expression from induction of a multicopy plasmid. Our hypothesis is thus that RI function is regulated by its proteolytic instability in the periplasm. The nature of the stabilization signal is unknown, but consideration of the molecular events during superinfection may provide a clue. It is thought that Imm, a small cytoplasmic membrane protein produced in quantity early in infection, causes secondary infections to fail, resulting in ectopic periplasmic localization of the capsid contents, which includes the 170-kb T4 chromosome and the more than 1,000 molecules of internal head proteins (1, 23, 24, 32). T4 "ghosts," emptied of DNA and internal proteins, do not cause LIN, although the ability to undergo tail contraction and

induce lethal channels in the cytoplasmic membrane is unaffected (18). The simplest model is that either the T4 DNA or the internal head proteins interfere in proteolysis of RI, thus indirectly activating the *r* system to block T holin. Recently Los et al. (22) reported that T4 wt, but not T4*rI*, exhibited delayed lysis in slow-growing cells cultured in chemostats, even when there were insufficient free phage to effect LIN by superinfection. This may reflect a significant stabilization of RI due to cellular responses to slow chemostat growth conditions rather than to the ectopic localization of the contents of superinfecting phage. In any case, experiments to test our model and address other unanswered questions about T4 lysis and LIN that arose decades ago during the classical era of the Delbrück "Phage Church" (5, 7), including the roles of the intensively studied *rIIAB* genes and also *rIII*, will be presented elsewhere.

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