

# RinA controls phage-mediated packaging and transfer of virulence genes in Gram-positive bacteria

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## ABSTRACT

Phage-mediated transfer of microbial genetic elements plays a crucial role in bacterial life style and evolution. In this study, we identify the RinA family of phage-encoded proteins as activators required for transcription of the late operon in a large group of temperate staphylococcal phages. RinA binds to a tightly regulated promoter region, situated upstream of the *terS* gene, that controls expression of the morphogenetic and lysis modules of the phage, activating their transcription. As expected, *rinA* deletion eliminated formation of functional phage particles and significantly decreased the transfer of phage and pathogenicity island encoded virulence factors. A genetic analysis of the late promoter region showed that a fragment of 272 bp contains both the promoter and the region necessary for activation by RinA. In addition, we demonstrated that RinA is the only phage-encoded protein required for the activation of this promoter region. This region was shown to be divergent among different phages. Consequently, phages with divergent promoter regions carried allelic variants of the RinA protein, which specifically recognize its own promoter sequence. Finally, most Gram-

positive bacteria carry bacteriophages encoding RinA homologue proteins. Characterization of several of these proteins demonstrated that control by RinA of the phage-mediated packaging and transfer of virulence factor is a conserved mechanism regulating horizontal gene transfer.

## INTRODUCTION

Horizontal gene transfer (HGT) between microorganisms has a great impact on the evolution of bacterial pathogens. Phages, plasmids and pathogenicity islands have all been recognized as mobile carriers of virulence-associated gene clusters. In HGT between bacteria, phages play important roles by carrying accessory virulence factors and by acting as gene transfer vehicles. Both functions are well documented in *Staphylococcus aureus* phages. Many clinically relevant toxins from staphylococci, including Panton–Valentine leukocidin (PVL), staphylokinase, enterotoxin A and exfoliative toxin A, are phage-encoded [recently reviewed in (1)]. In addition, a number of staphylococcal superantigens are encoded on a family of pathogenicity islands (SaPIs) that exploit certain helper bacteriophages for high frequency horizontal transfer. In the absence of a helper phage, SaPIs reside stably in the chromosomes of their host bacteria under the control of the master repressor *Stl* (2). Following helper phage infection, SaPIs are

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specifically de-repressed by phage-encoded antirepressors (3) (M.D. Harwich *et al.*, in preparation), leading to their excision from the chromosome and their autonomous replication (4). The SaPI-encoded small terminase subunit then directs encapsidation of the replicated SaPI DNA (5) in phage-like particles composed of phage virion proteins (6,7) but with smaller capsids that accommodate the SaPI genome (generally 15–18 kb) while excluding the larger genome of the helper phage (8,9).

Relatively little is known about the biology of staphylococcal phages, despite their relevance in the pathogenic process of *S. aureus* and the availability of more than a hundred *S. aureus* phage and prophage genome sequences in public databases. Most known staphylococcal phages are temperate, tailed bacterial viruses belonging to the family *Siphoviridae*. The genomes of these phages are organized in discrete functional modules, including those for lysogeny, DNA replication, phage assembly, DNA packaging and host cell lysis. Like other bacteriophages, these staphylococcal phages are predicted to exert temporal control of gene expression during infection of a sensitive host, or during induction from a lysogenic strain. This temporal transcription of phage genes is expected to be divided into at least three stages; early (expression of the lysogeny and control modules), middle (replication) and late (packaging, assembly and lysis modules).

In this work, we have characterized genes controlling expression of the phage late genes. Previous results from our group, using lysogenic, SaPI-positive strains, demonstrated that in the absence of phage induction, no phage or SaPI particles were produced and there was no horizontal transfer of the SaPI-encoded virulence factors. Since SaPIs are encapsidated in phage-encoded particles, we expected that control of expression of the morphogenetic and lysis modules of the staphylococcal phages is an essential step in phage-mediated transfer of virulence factors encoded on these mobile genetic elements (MGE) as well as for the transfer of toxins carried on the phage genomes themselves. In this study we have examined transcriptional control of the highly conserved morphogenetic gene cluster present in phages  $\phi$ 11 and 80 $\alpha$ , which serve as helpers for some of the well-studied SaPIs, as well as in the clinically relevant phage  $\phi$ SLT, which encodes the PVL toxin. Phage  $\phi$ 11 mobilizes SaPIbov1 while 80 $\alpha$  induces several SaPIs, including SaPI1, SaPI2, SaPIn1, SaPIbov1 and SaPIbov2 (10). We demonstrate that the phage *rinA* gene, previously reported to be a regulator of *int* transcription (11), is actually an activator of phage late gene transcription and not of *int*. Phages lacking *rinA* did not generate either phage or SaPI functional particles, and were defective in horizontal transfer of MGE-encoded virulence factors. Furthermore, since RinA homologs are also present in phages from other clinically relevant Gram-positive bacteria, including *Streptococcus suis*, *Clostridium botulinum*, *Listeria monocytogenes* and *Enterococcus faecalis*, we extended our results by demonstrating that these RinA homologues are functionally identical to those characterized from staphylococci, which indicates that control of phage morphogenetic genes by RinA is a widespread mechanism used by phages infecting Gram-positive bacteria.

## MATERIAL AND METHODS

### Bacterial strains and growth conditions

Bacterial strains used in these studies are listed in Supplementary Table S1. Bacteria were grown at 37°C overnight on Trypticase Soy (TSA) agar medium, supplemented with antibiotics as appropriate. Broth cultures were grown at 37°C in Trypticase Soy Broth (TSB) broth with shaking (240 rpm).

For prophage induction, bacteria were grown in TSB to OD<sub>540</sub> = 0.4 and induced by adding mitomycin C (2 µg/ml). Cultures were grown at 32°C with slow shaking (80 rpm). Lysis usually occurred within 3 h. Samples were removed at various time points after phage induction, and standard SDS minilysates were prepared and separated on 0.7% agarose gels, as described previously (12,13). Standard procedures were used for preparation and analysis of phage lysates, lysogens and transduction in *S. aureus*, as described previously (12).

### DNA methods

General DNA manipulations were performed by standard procedures (14,15). Oligonucleotides used in this study are listed in Supplementary Table S3. Labeling of the probes and DNA hybridization were performed according to the protocol supplied with the PCR-DIG DNA-labeling and chemiluminescent detection kit (Roche). To produce the strains carrying mutant prophages, allelic exchange was performed using derivatives of plasmid pMAD (16) carrying the desired mutations, as described previously (8). Plasmid constructs (Supplementary Table S2) were prepared by cloning PCR products obtained from oligonucleotide primers as listed in Supplementary Table S3. All constructs were sequenced by the Institute core sequencing laboratory.

### Enzyme assays

$\beta$ -Lactamase assays, using nitrocefin as substrate, were performed as described (17), using a Thermomax (Molecular Devices) microtiter plate reader. Cells were obtained in exponential phase.  $\beta$ -Lactamase units are defined as (V<sub>max</sub>)/OD<sub>650</sub>.

### Real-time quantitative PCR

Total *S. aureus* RNA was prepared using the Fast RNA-Blue kit (Bio101) according to the manufacturer's instructions. Two micrograms of each RNA were subjected, in duplicate, to DNase I (Invitrogen) treatment for 30 min at 37°C. The enzyme was inactivated at 65°C in the presence of EDTA. To verify the absence of genomic DNA in every sample, the RNA duplicates were reverse transcribed in the presence and absence of M-MLV Reverse Transcriptase (Invitrogen). All preparations were purified using QIAquick PCR purification kit (Qiagen). Twenty-five nanograms of each reaction product was used for a real-time quantitative PCR using the iCycler machine (Bio-Rad) and the LC-DNA Master SYBR Green I mix (Biorad). The different genes were amplified using oligonucleotides listed in Supplementary Table S2. The *gyrB* transcripts that are constitutively expressed were amplified

as an endogenous control. The level of expression of the different genes were normalized with respect to *gyrB* expression. Only samples with no amplification of *gyrB* in the minus reverse transcriptase aliquot were included in the study. To monitor the specificity, the final PCR products were analyzed by melting curves and electrophoresis. In each experiment, all the reactions were performed in triplicate. The relative transcriptional levels within distinct experiments were determined by using the  $2^{-\Delta\Delta CT}$  method (18). The results show the average  $\pm$  SEM of at least four independent experiments.

### Purification of RinA protein

The *rinA* gene was amplified by PCR using primers orf28phi11-5cX and orf28phi11-7mB (Supplementary Table S3), digested with *Xba*I and *Bam*HI, and cloned into plasmid pGEX-4T-1 (GE Healthcare). Expression and purification of the GST-tagged fusion RinA protein was performed following the instructions of the manufacturer. The purity of the purified GST-tagged RinA fusion protein was confirmed by sodium dodecyl sulphate (SDS) gels stained with Commassie Brilliant Blue R-250. The purified protein was found to be more than 98% pure in an SDS-12% polyacrylamide gel. The concentration of the purified proteins was determined by the Bradford protein assay (Bio-Rad, Hercules, CA), using bovine serum albumin as the standard.

### Mobility shift assays

Electrophoretic mobility shift assays (EMSA) were performed as described before (3,5) using purified RinA protein and a DIG-labeled DNA fragment obtained by PCR using the oligonucleotides listed in Supplementary Table S3.

### Southern blot analysis of phage late promoters

RNA samples isolated during a time course of 80 $\alpha$  infection were pooled to yield 100  $\mu$ g of total RNA which was concentrated to 12  $\mu$ l using an RNeasy MinElute column (Qiagen). This RNA was then used as a substrate for vaccinia virus capping enzyme, using the ScriptCap m7G Capping System (Epicenter Biotechnologies). The RNA was heated at 65°C for 10 min, placed immediately on ice, and then incubated at 37°C for 1 h in a reaction containing 1 $\times$  reaction buffer (provided by the manufacturer), 1 mM GTP (including 1 mCi of  $\alpha$ -<sup>32</sup>P GTP), 0.1 mM SAM, 50 U of RNase inhibitor and 25 U of capping enzyme. The labeled RNA was purified using a RNeasy Minelute column, and an aliquot was checked on a gel to ensure that most of the labeled RNA was small enough (>200 bp) to hybridize only to fragments containing the initial transcribed region. The RNA probe was incubated at 70°C for 10 min, and added to 10 ml of UltraHyb (Ambion) for hybridization to Southern blots. A positively charged nylon membrane (ICN) containing restriction fragments of 80 $\alpha$  DNA that had been resolved by agarose gel electrophoresis was prepared by capillary transfer using standard methods (15) and pre-hybridized in UltraHyb for 1 h at 42°C prior to addition of the probe. After hybridization overnight at 42°C, membranes were

washed twice at 42°C for 10 min in 2 $\times$  SSC, 0.1% SDS and twice at 42°C for 10 min in 0.1 $\times$  SSC, 0.1% SDS prior to exposure to a phosphor screen (Molecular Dynamics).

### Rapid amplification of cDNA 5'-ends

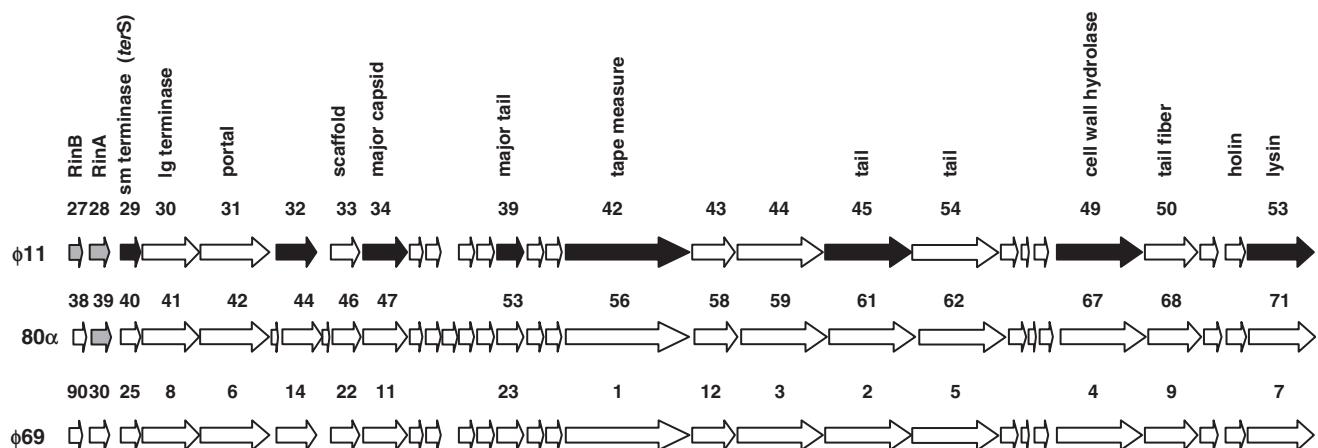
Amplification of the *terS* cDNA 5'-end of  $\phi$ 11 was performed using the 5'/3' RACE kit (Roche), according to the manufacturer's protocol. First-strand cDNA synthesis was performed using the oligo ORF29-phi11-sp1c (Supplementary Table S3). The cDNA mixtures were amplified by PCR using the oligo(dT) anchor primer and the gene-specific primers ORF29-phi11-sp2c and ORF29-phi11-sp3c (Supplementary Table S3). The PCR products were purified from 1.2% agarose gels and subjected to DNA sequence analysis. The 5'-end of 80 $\alpha$  *terS* was amplified using the FirstChoice RLM-RACE kit (Ambion) according to the manufacturer's instructions, after treatment of RNA with Terminator enzyme (Epicentre Biotechnologies) to degrade fragments containing 5' monophosphate termini. First strand cDNA synthesis was performed using random decamers, after ligation of the 5' rapid amplification of cDNA 5'/3' ends (RACE) adapter. Following cDNA synthesis, nested PCR was performed using Pfu Turbo (Stratagene) with *terS* specific primers MH22 and MH23 (Supplementary Table S3) and the 5' outer and inner adapter specific primers provided with the kit. Amplicons from the MH22/inner adapter primer reaction were ligated into *Nru*I-digested pBR322 and individual clones were sequenced.

## RESULTS

### Deletion of *rinA* eliminates production of both phage and SaPI particles

The virion proteins of  $\phi$ 11 and 80 $\alpha$ , which are involved in the production of both phage and SaPI particles (6,7,19), are encoded by a cluster of genes that are all oriented in the same direction on the phage genome (Figure 1). We expected phage-encoded factor(s) to be required for expression of these morphogenetic genes. Two attractive candidates for late gene regulation were RinA and RinB, homologous of which are found in the vast majority of *S. aureus* *Siphoviridae* and which are encoded by genes immediately upstream of the morphogenetic gene cluster. These two genes had been reported previously to be involved in activation of expression of the cloned phage  $\phi$ 11 *int* gene (11), consistent with a regulatory function. To analyze their possible role as regulators of the phage morphogenetic genes, we separately inactivated either *rinA* or *rinB* in a  $\phi$ 11 prophage by constructing in-frame deletions. We also introduced SaPIbov1 containing a tetracycline marker inserted in the *tst* locus (SaPIbov1 *tst::tetM*) into the strains carrying the prophage mutants, in order to examine effects on production of SaPIbov1 transducing particles as well as infectious phage. The different phage mutants (with or without SaPIs) were analyzed for definable stages of the phage and SaPI life cycles following induction with mitomycin C.

Deletion of *rinA* eliminated or significantly reduced the production of functional phage or SaPI particles,



**Figure 1.** Location of the genes controlled by RinA. Partial genetic maps of  $\phi$ 11 (GenBank accession number AF424781), 80 $\alpha$  (accession number DQ517338) and  $\phi$ 69 (accession number AY954951), showing the location of *rinA* and the genes under *rinA* control. The three phages share 96% nucleotide identity across this entire region. Arrows indicate predicted Open Reading Frames (ORFs), numbered as annotated. Gray arrows indicate the regulatory genes deleted in this study, while black arrows indicate genes for which *rinA* dependence was directly demonstrated by qRT-PCR.

as assayed by plaque formation or transduction, respectively (Table 1). Furthermore, there was no lysis of the *rinA* mutant after induction, suggesting that these mutants were defective in expression of the lysis module. The *rinA* mutant was not impaired in either  $\phi$ 11 or SaPIbov1 DNA replication; Southern blot analysis of minilysates prepared 60 min after induction showed that the phage and the SaPI signals in the bulk DNA were amplified to essentially the same degree in the mutant as in the wild-type strains (Figure 2). However, the *rinA* mutant failed to produce a linear SaPI extrachromosomal band. This is consistent with an encapsidation defect, as the SaPI band is believed to arise from the release of packaged SaPI DNA by disruption of intracellular SaPI capsids (5,6). Similar results were obtained following deletion of the *rinA* gene from 80 $\alpha$  (Table 1), which was expected because both *rinA* (Supplementary Figure S1) and the entire morphogenetic gene cluster of 80 $\alpha$  and  $\phi$ 11 (Figure 1) are essentially identical. In order to determine the generality of this mechanism, we also constructed a *rinA* mutant of phage  $\phi$ SLT, which encodes a RinA homolog that is only 23% identical to that of  $\phi$ 11 (Supplementary Figure S1), although similar to RinA proteins encoded by other staphylococcal phages (data not shown). We used a detoxified derivative of phage  $\phi$ SLT (20) containing a tetracycline-resistant marker (*terM*) inserted in the PVL locus (21). This mutant was also deficient in generating functional phage particles (Table 1), and in lysing the bacteria after prophage induction.

To confirm that the observed phenotypes were due to the *rinA* mutations, plasmids pJP740 (pCN51-*rinA* $_{\phi$ 11}), and pJP838 (pCN51-*rinA* $_{\phi$ SLT}), carrying the *rinA* genes from  $\phi$ 11 and  $\phi$ SLT, respectively, under the control of an inducible promoter, were introduced into strains carrying the corresponding *rinA* mutant prophage. Complementation with plasmid-encoded RinA restored packaging, lysis and production of viable phage (Table 1), confirming its crucial role in phage gene expression. Similarly, complementation of SaPI-positive  $\phi$ 11

and 80 $\alpha$  *rinA* lysogens with plasmid pJP740 restored SaPI packaging and transfer (Table 1).

Mutation of *rinB* had no effect on either phage production or transfer of SaPI (data not shown). Although an earlier report implicated RinB in the expression of the  $\phi$ 11 *int* gene (11), our results suggest that expression of integrase at levels needed for prophage induction must be RinB-independent. Regulation of *int* thus appears to be more complex than initially suggested by the report of Ye *et al.* (11), and is the subject of further investigation. In addition, RinB does not appear to play a role in regulation of late gene expression.

#### RinA activates transcription of the packaging, assembly and lysis genes

The phenotype of the *rinA* mutants suggested that RinA might control phage and SaPI packaging through expression of the phage morphogenetic genes. The mRNA levels of several  $\phi$ 11 late genes were measured by quantitative real-time PCR at various times (0, 30 and 45 min) after mitomycin C induction, comparing expression levels in the wild-type phage and the *rinA* mutant. As shown in Figure 3, the *rinA* mutation resulted in a significant ( $P < 0.05$ ) decrease in the expression of genes involved in DNA packaging, capsid assembly and cell lysis. Similar results were obtained for the genes encoding the major capsid protein and several components of the phage tail (data not shown). These results indicate that RinA is required for transcription of the phage-encoded packaging and lysis modules, and also confirm the expected temporal expression of these genes.

#### Identification of a RinA-inducible promoter upstream of *terS*

Based on genome organization, we predicted the existence of at least one promoter directly controlled by RinA and located just upstream of the terminase small subunit gene (*terS*; ORF29  $\phi$ 11), the first gene in the morphogenetic gene cluster. To determine the transcription start site

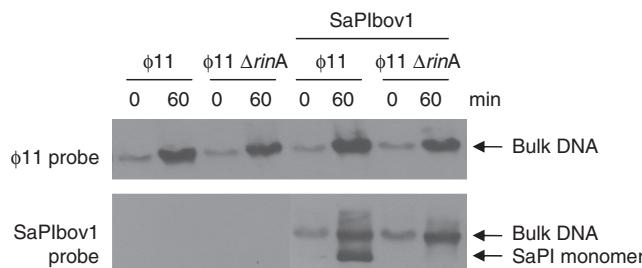
**Table 1.** Effect of phage mutations on phage titre and SaPI transfer<sup>a</sup>

Donor strain	φ	Plasmid	SaPI	Phage titre <sup>b</sup>	Transduction titre <sup>c</sup>
RN451	φ11 wt	—	—	1.3 × 10 <sup>9</sup>	—
JP1794	φ11 wt	—	SaPIbov1	1.4 × 10 <sup>7</sup>	1.6 × 10 <sup>8</sup>
JP4028	φ11 ΔrinA	—	—	<10	—
JP4221	φ11 ΔrinA	pCN51-rinA <sub>φ11</sub>	—	2.4 × 10 <sup>8</sup>	—
JP4128	φ11 ΔrinA	—	SaPIbov1	<10	8.0 × 10 <sup>3</sup>
JP5961	φ11 ΔrinA	pCN51-rinA <sub>φ11</sub>	SaPIbov1	2.2 × 10 <sup>6</sup>	2.4 × 10 <sup>7</sup>
RN10359	80α wt	—	—	2.9 × 10 <sup>10</sup>	—
JP3603	80α wt	—	SaPIbov1	6.9 × 10 <sup>8</sup>	7.8 × 10 <sup>7</sup>
JP3602	80α wt	—	SaPI1	4.5 × 10 <sup>8</sup>	8.2 × 10 <sup>8</sup>
JP4717	80α ΔrinA	—	—	<10	—
JP5418	80α ΔrinA	pCN51-rinA <sub>φ11</sub>	—	6.1 × 10 <sup>8</sup>	—
JP5293	80α ΔrinA	—	SaPIbov1	<10	770
JP5294	80α ΔrinA	—	SaPI1	<10	22
JP5419	80α ΔrinA	pCN51-rinA <sub>φ11</sub>	SaPIbov1	9.0 × 10 <sup>7</sup>	4.0 × 10 <sup>7</sup>
JP5420	80α ΔrinA	pCN51-rinA <sub>φ11</sub>	SaPI1	2.8 × 10 <sup>7</sup>	6.9 × 10 <sup>7</sup>
JP5011	φSLT wt	—	—	1.7 × 10 <sup>5</sup>	8.7 × 10 <sup>4</sup>
JP6895	φSLT ΔrinA	—	—	<10	<10
JP6391	φSLT ΔrinA	pCN51-rinA <sub>φSLT</sub>	—	9.0 × 10 <sup>4</sup>	7.7 × 10 <sup>3</sup>
JP7188	φ11-φ69 chimera	—	—	<10	—
JP7242	φ11-φ69 chimera	—	SaPIbov1	<10	1.2 × 10 <sup>3</sup>
JP7218	φ11-φ69 chimera	pCN51-rinA <sub>φ69</sub>	—	ND	—
JP7243	φ11-φ69 chimera	pCN51-rinA <sub>φ69</sub>	SaPIbov1	ND	4.2 × 10 <sup>6</sup>

<sup>a</sup>The means of results from three independent experiments are presented. Variation was within ±5% in all cases.

<sup>b</sup>Pfu/ml of induced culture, using RN4220 (or pCN51-complemented RN4200) as recipient.

<sup>c</sup>Transductants/ml of induced culture, using RN4220 as recipient.



**Figure 2.** Characterization of the φ11 *rinA* mutant. Southern blot of φ11 wt and *rinA* mutant lysates from strains with or without SaPIbov1 *tst::tetM*. Samples were taken before or 60 min after MC induction, minilysates were prepared, the DNA was separated on agarose and blotted with a phage- or SaPIbov1-specific probe, as indicated. The upper band is ‘bulk’ DNA, including chromosomal, phage and replicating SaPI DNA; the lower band is SaPI linear monomers released from phage heads.

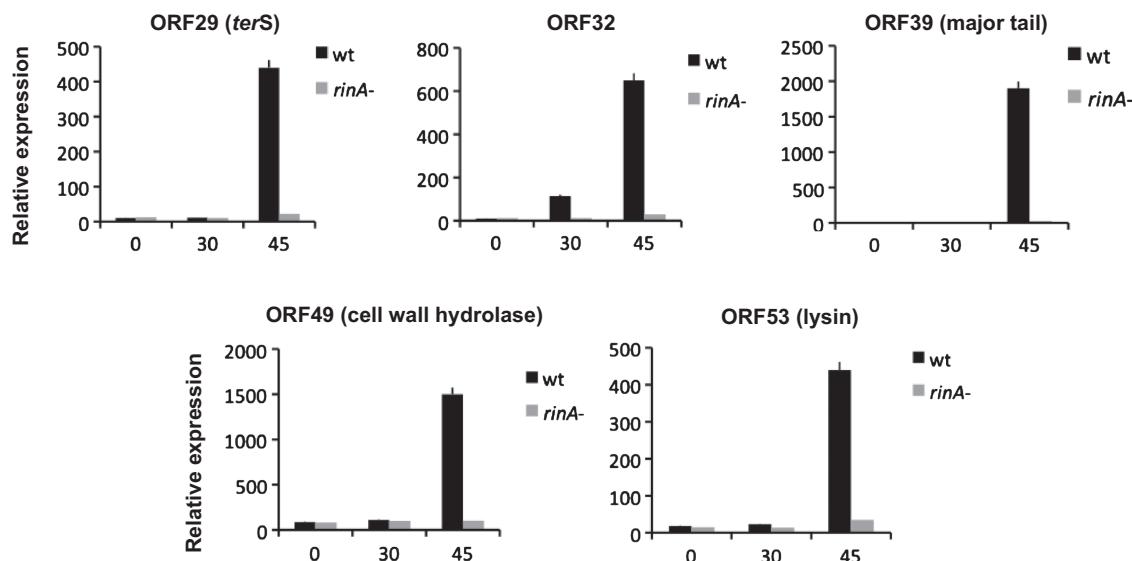
and locate this putative promoter, 5' RACE analysis was performed with total RNA isolated after induction of the φ11 prophage in strain RN451. A *rinA*-dependent 5'-end was identified in RNA isolated 60 min after MC induction of the wild-type (wt) strain RN451, which mapped to a C residue located 23 bp upstream of the *terS* ATG initiation codon (Supplementary Figure S2). In the case of 80α late mRNA, the 5'-end was mapped to the adjacent G residue 24 bp upstream of the *terS* gene.

To determine whether the observed 5'-end corresponded to a promoter that was indeed regulated by RinA, a 425 bp fragment spanning the *rinA-terS* junction was fused to the β-lactamase reporter gene present in plasmid pCN41, generating plasmid pJP742. This plasmid was introduced into strains RN451 (φ11 lysogen) and JP4028 (RN451 ΔrinA), and, after prophage induction, the transcription

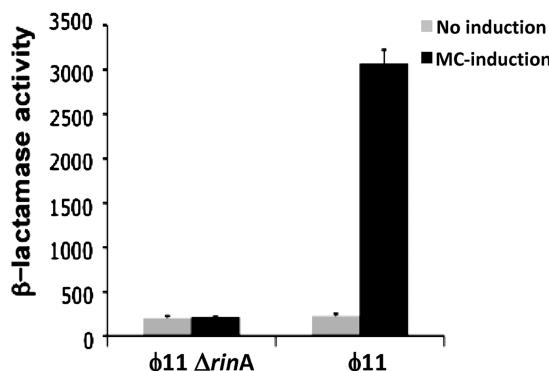
of the β-lactamase reporter was analyzed. As shown in Figure 4, β-lactamase expression increased significantly after induction of the wt strain RN451 but not in its derivative *rinA* mutant, clearly confirming the existence of a promoter regulated by RinA in this region of the φ11 genome.

#### RinA is sufficient to activate *ter* expression

We next examined whether expression of RinA was enough to induce expression of the *terS* gene in the absence of other phage-encoded proteins. To test this we made use of plasmid pCN42, which contains a *Pcad* inducible promoter and a β-lactamase reporter (Figure 5). We introduced into this plasmid a PCR fragment containing the *rinA* coding sequence (expression of which depends on the *Pcad* promoter present in the plasmid), the *terS* promoter region, and the 5' coding region of the *terS* gene, which was fused to the β-lactamase reporter. The resulting plasmid, pJP743, is illustrated in Figure 5. As a control, we amplified this region using as a template the DNA from the φ11 *rinA* mutant, generating plasmid pJP744, which does not express RinA. These plasmids were introduced into strain RN4220, and the expression of the β-lactamase reporter was measured. As shown in Figure 5, only the plasmid expressing RinA also expressed the β-lactamase reporter, confirming the previous results and indicating that RinA is the only phage-encoded protein required for the expression of *terS*. Furthermore, the lack of β-lactamase expression from the *rinA* mutant plasmid indicates the presence of a transcriptional terminator between the *rinA* and *terS* genes. One potential sequence in the region with the characteristics of



**Figure 3.** RinA controls expression of the morphogenetic and lysis clusters of  $\phi$ 11. Real-time quantification of expression of different genes in  $\phi$ 11 wild type and the *rinA* mutant at different times (0, 30 or 45 min) after MC induction of the phage lytic cycle. Expression was normalized to *gyrB*.



**Figure 4.** Effect of *rinA* on transcription of *terS*.  $\text{RN}451$  or its derivative *rinA* mutant strain  $\text{JP}4028$  containing plasmid  $\text{pJP}742$  (*terS*-*blaZ* transcriptional fusion) were assayed 90 min after prophage induction for  $\beta$ -lactamase activity under standard conditions, as described in 'Material and Methods' section. Samples were normalized for total cell mass ( $\beta$ -lactamase units/ $\text{OD}_{650}$ ).

a factor-independent terminator is indicated in Supplementary Figure S2.

#### Specificity of RinA activation

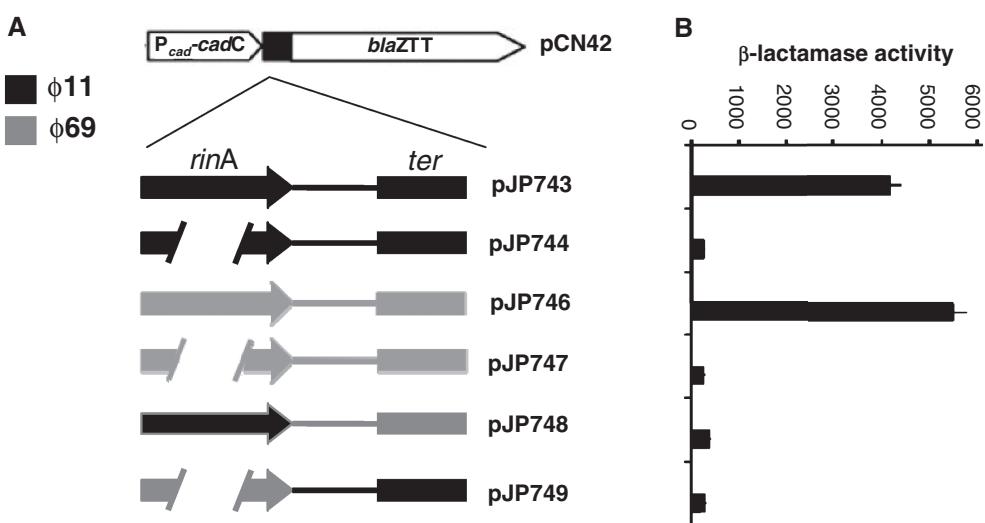
The  $\phi$ 69 RinA homolog has limited similarity to that of  $\phi$ 11 (Supplementary Figure S1), although the packaging module of both phages is basically identical (Figure 1). Using the same  $\beta$ -lactamase expression system described above, we showed that  $\text{RinA}_{\phi 69}$  was also necessary and sufficient to activate  $\phi 69$  *terS* expression (plasmids pJP746, pJP747; Figure 5). We next tested for possible cross-reactivity between the  $\phi$ 11 and  $\phi$ 69 RinA proteins by determining whether either could activate *terS* expression from the other phage. Plasmid pJP748 carries the coding region of  $\text{rinA}_{\phi 11}$  under the control of the inducible promoter present in pCN42, and the promoter region and *terS* 5' coding region from  $\phi$ 69 fused to *blaZ*. In contrast,

plasmid pJP749 carries the coding region of  $\text{rinA}_{\phi 69}$ , and the promoter region and partial *terS* gene from  $\phi$ 11. As shown in Figure 5, the two different RinA proteins uniquely activated expression of their cognate *terS* genes. Since the sequences 5' to *terS* are highly similar until  $\sim 120$  bp upstream of the presumptive transcription start and then diverge (Supplementary Figure S2B), we speculate that the different RinA proteins recognize these divergent regions to regulate phage late gene expression.

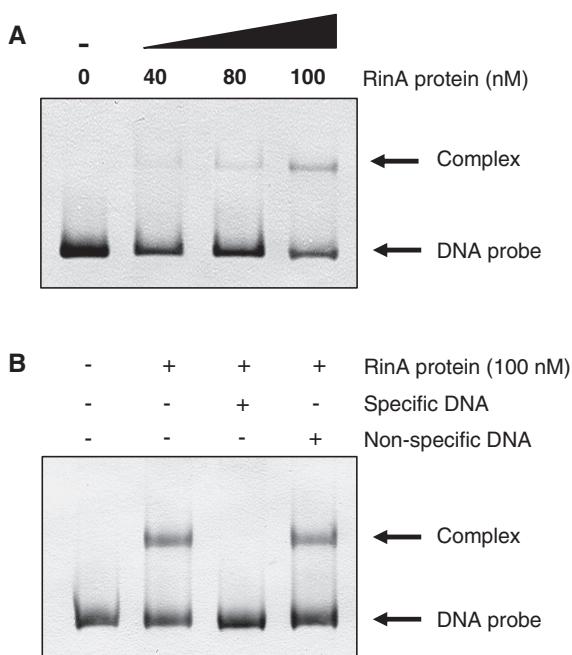
#### RinA binds to the *terS* promoter

The results described above suggested that RinA should bind specifically to the *terS* promoter region. To test this, GST-tagged  $\phi$ 11 RinA was expressed in *Escherichia coli*, purified, and incubated with an end-labeled 272-bp PCR fragment spanning the entire presumptive *terS* promoter region (see Supplementary Figure S2 for the sequence of this region). Complexes were examined by electrophoretic mobility shift assays. As shown in Figure 6A, the recombinant RinA protein retarded mobility of the *terS* promoter fragment in a dose-dependent manner. Detectable binding to the labeled fragment was eliminated in the presence of an excess (500-fold) of unlabeled *terS* fragment, but not when a 500-fold excess of non-specific unlabeled DNA fragment was used as a competitor (Figure 6B). This confirms the specific binding of RinA protein to the *terS* promoter region, consistent with its role as an activator of *terS* transcription.

To examine whether the divergent part of the region was involved in the observed DNA-binding activity of RinA, smaller DNA fragments lacking 87 or 50 bp from the 5'-end of this 272 bp fragment were constructed (Supplementary Figure S2). When EMSA was performed using these DNA fragments, no protein-DNA binding was detected (data not shown), consistent with a



**Figure 5.** Specificity of RinA proteins. (A) Schematic representation of the different *blaZ* transcriptional fusions. (B) Derivatives of strain RN4220 containing each of the indicated plasmids were assayed at mid-exponential phase for β-lactamase activity under standard conditions. Samples were normalized for total cell mass (β-lactamase units/OD<sub>650</sub>).



**Figure 6.** Binding of RinA to the *terS* promoter. (A) Electrophoretic mobility of a DIG-labeled promoter fragment was measured in the presence or absence of increasing amounts of purified φ11 RinA protein, as indicated on the top line. (B) In the competition assays, 500-fold excesses of specific- or non-specific unlabeled DNA fragments were added, as indicated. In all cases, the concentration of the DIG-labeled probe was 21 nM.

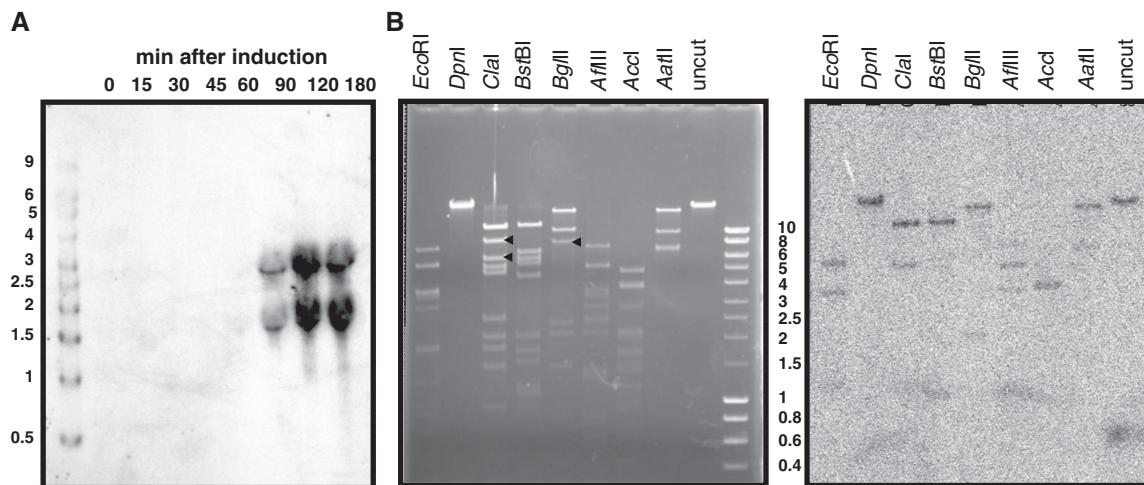
requirement for virtually all of this upstream divergent region in RinA binding.

#### Characterization of the *rinA*-dependent transcription unit

The *terS* gene lies just downstream of *rinA*, and at the beginning of the phage morphogenetic gene cluster. All

of the morphogenetic genes are encoded on the same strand, with little intergenic space between them, suggesting that they might comprise a single large transcription unit. In an attempt to characterize the phage transcription units, independent Northern blot analyses were performed on RNA isolated during both 80 $\alpha$  and φ11 infections. In both cases, although specific hybridization was obtained at appropriate time points, only small RNAs in the 2–4 kb size range could be detected, using probes from several different genes across this region. A representative example is shown in Figure 7A. This suggests that either there are multiple transcription start sites throughout the late gene cluster or that the RNA is rapidly processed. Multiple transcription start sites should yield multiple RNAs containing 5' triphosphate ends. To estimate the number of promoters present in the phage genome, Southern blot analysis of 80 $\alpha$  DNA was performed with pooled phage RNA collected at various times after infection and labeled specifically at the 5' triphosphate termini arising from initiation of transcription. This analysis does not allow unambiguous identification of unique promoter-containing fragments, but did indicate that the total number of promoters in the phage genome is relatively small (Figure 7B). Furthermore, several unique large restriction fragments that included only late region DNA downstream of *terS* were not detected in this analysis, consistent with the hypothesis that there is a single promoter for the late genes.

To investigate further the transcription of the morphogenetic genes, we isolated RNA late in infection with 80 $\alpha$ , converted it to cDNA and used that cDNA in endpoint RT-PCR primer pairs flanking intergenic regions throughout the late gene cluster, from terminase through the lysis genes. A product was obtained with all primer pairs from *terL* to ORF69 (data not shown). The presence of RNA spanning each of the late gene intergenic regions also suggests that these genes are all cotranscribed. The



**Figure 7.** Detection of transcription start sites in the 80 $\alpha$  genome. (A) Northern blot of RNA isolated at the indicated time points after MC induction of an 80 $\alpha$  lysogen and probed with a biotinylated *terS* oligonucleotide. Sizes in kilobase of the markers (Riboladder Long, Bioline) are shown on the left. (B) Agarose gel (left) and Southern blot (right) of 80 $\alpha$  virion DNA, digested with the indicated restriction enzymes and probed with total RNA that was isolated from infected cells at various time points, pooled, and 5'-end-labeled at triphosphate termini using vaccinia virus capping enzyme. Sizes in kilobase of the markers (HyperLadder I, Bioline) are indicated to the right of the gel. Black arrowheads on the gel denote examples of unique large fragments from the late region that are not detected in the blot, but to which hybridization would be expected if there were additional transcription start sites downstream of *terS*.

alternative explanation for the endpoint RT-PCR results, i.e. the presence of multiple promoters within the late gene coding sequences reading through into adjacent genes, is not consistent with the absence of transcription start sites in the late region revealed by the Southern blot analysis.

We did not obtain amplification of the intergenic regions between ORF69 and holin or between holin and lysin, although the genomic DNA in this region was readily amplified with the same primer pairs (data not shown). The intergenic regions between ORF69 and holin and between holin and lysin are 58 and 12 nt, respectively, making it unlikely that new transcription initiates in these regions. We were unable to demonstrate the existence of a promoter in this region of the genome by 5' RACE (data not shown). It is possible that RNA processing explains the lack of PCR amplification across these two intergenic regions, or that RNA secondary structure interfered with cDNA synthesis.

We felt it necessary to further investigate the possibility of an additional promoter in this region, however, because studies on  $\phi$ Sa3ms, a related temperate siphovirus that carries a gene for staphylococcal enterotoxin SEA just upstream of the lysis genes, suggested that this phage had a promoter near the end of the tail gene cluster (22). We replaced the *terS* upstream region in strain RN451, which is lysogenic for  $\phi$ 11, with the corresponding region from  $\phi$ 69, generating strain JP7188. We also introduced SaPIbov1 *tst::tetM* into this strain, generating strain JP7242. We predicted that if an additional RinA-dependent promoter controls the lysis module, these strains will lyse after MC-induction, although no functional particles will be generated, since the RinA from  $\phi$ 11 will not bind to the new RinA $_{\phi$ 69}-binding region controlling *terS* expression. In contrast, if all genes are cotranscribed from the promoter situated 5' of the *terS* gene, these

strains not only will fail to produce functional SaPI or phage particles, but also will not lyse. As shown in Table 1, the latter was the case; no lysis was observed when the prophage carried the heterologous *rinA* binding region upstream of *terS*. In addition, qRT-PCR demonstrated that the expression of genes from the packaging and lysis modules were significantly reduced compared with wild-type strains (data not shown). Finally, strains JP7188 and JP7242 were complemented with plasmid pJP741, which overexpresses the  $\phi$ 69 RinA. The complemented strains lysed and produced functional SaPI particles after MC-induction (Table 1). Taken together, the simplest interpretation of all of these results is that there is a single late gene transcription unit, initiating from the RinA-dependent promoter just upstream of *terS*.

#### RinA does not control *int* expression

As discussed earlier, *rinA* was initially identified as a positive regulatory gene required for expression of the cloned  $\phi$ 11*int* gene (11). To investigate this further, we analyzed *int* expression in  $\phi$ 11 wt and *rinA* mutant strains, using qRT-PCR. Expression of *int* was not affected in the *rinA* mutant (Supplementary Figure S3), suggesting that the RinA protein does not have a direct role controlling the expression of *int*, at least under conditions of prophage induction.

To confirm this, we analyzed the activity of the Int protein in the *rinA* mutant. Previous work had identified Int as essential for phage integration and excision (23). We predicted that prophage excision would be reduced in the *rinA* mutant if RinA is required for *int* expression. We measured by qPCR the excision activities of prophages  $\phi$ 11,  $\phi$ 11  $\Delta$ *rinA* and  $\phi$ 11  $\Delta$ *int* (as a control), using primers hybridizing to the regions flanking the  $\phi$ 11 *att*<sub>B</sub>

site. As expected, excision of the  $\phi 11$  *rinA* mutant was similar to wt, while no excision was observed in the  $\phi 11$   $\Delta$ *int* mutant (data not shown). This confirms the previously reported requirement for Int in prophage excision as well as the lack of involvement of RinA in regulation of *int* expression during this process.

### RinA does not control SaPI-encoded operon I expression

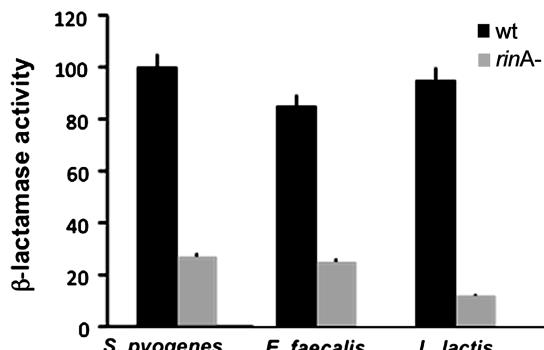
We have previously reported that SaPI packaging depends on expression of the SaPI-encoded homologue of the phage terminase small subunit (*terS*). In addition, SaPIs remodel assembly of the phage capsid proteins to generate capsids one-third the size of the helper phage capsids, which accommodate their smaller genomes while excluding complete helper phage genomes. This requires two SaPI genes, *cp1* and *cp2*, which are adjacent in a six-gene LexA-regulated operon, operon I, that also encodes the SaPI-specific *terS* (5). In order to determine if RinA controls expression of SaPIbov1 operon I, we analyzed SaPIbov1 *terS* expression in  $\phi 11$  wt and *rinA* mutant strains, using qRT-PCR. Expression of SaPIbov1 *terS* was not impaired in the *rinA* mutant, even under conditions where LexA-dependent SaPI operon I expression was blocked by mutation (data not shown), suggesting that the RinA protein does not have a direct role controlling the expression of SaPI-encoded operon I.

### RinA homologues control packaging in phages from other Gram-positive bacteria

RinA from  $\phi 11$  is the archetype for a widespread family of conserved phage proteins. Since RinA homologs are present in phages from a number of other Gram-positive bacteria (Table 2), we analyzed the possibility that the mechanism described here, implicating RinA in control of phage packaging and lysis, is a common one. To test the role of additional RinA homologs in expression of phage packaging genes, we introduced into plasmid pCN42, carrying the *blaZ* transcription fusions, fragments including the *rinA* gene, the putative promoter region located upstream of the *terS* gene, and the 5' region of the *terS* gene from prophages found in *E. faecalis*, *Streptococcus pyogenes* or *Lactococcus lactis* (Table 2), generating plasmids pJP535 (*E. faecalis*), pJP543 (*S. pyogenes*) and pJP564 (*L. lactis*). As a control, we also generated plasmids carrying in-frame deletions in each of the *rinA* homolog genes (plasmids pJP537, pJP544 and pJP565, respectively). We introduced these plasmids into *S. aureus* strain RN4220 and assayed expression of the  $\beta$ -lactamase reporter gene. Not only were the phage late promoter regions present in these plasmids recognized by the *S. aureus* RNA polymerase enzyme, but also expression of each was dependent upon the *rinA* homolog carried on the cloned fragment (Figure 8). This confirms that RinA activation of late transcription is a widespread strategy used to control packaging and lysis by phages infecting Gram-positive bacteria.

**Table 2.** RinA homologs in phages from Gram-positive bacteria

Species	Accession number
<i>Lactobacillus casei</i>	YP_001987048
<i>Lactobacillus reuteri</i>	ZP_03073841
<i>Bacillus thuringiensis</i> serovar <i>pakistani</i> str.	ZP_04123836
<i>Geobacillus</i> sp. <i>G11MC16</i>	ZP_03149736
<i>Lactobacillus plantarum</i>	ACT61868
<i>Enterococcus faecalis</i>	AAO81076
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	YP_003353517
<i>Clostridium thermocellum</i>	ABN53671
<i>Streptococcus suis</i>	ZP_03626078
<i>Clostridium botulinum</i>	EES48701
<i>Listeria monocytogenes</i>	ADB68097
<i>Streptococcus gallolyticus</i>	YP_003429885
<i>Clostridium</i> phage <i>phiSM101</i>	YP_699949
<i>Streptococcus equi</i> subsp. <i>equi</i>	YP_002747279
<i>Streptococcus pneumoniae</i>	ACA36251
<i>Lactococcus</i> phage <i>phiLC3</i>	AAS66802
<i>Streptococcus pyogenes</i>	ABF36520
<i>Lactobacillus jensenii</i>	ZP_05861716
<i>Bacillus cereus</i>	ZP_04240708
<i>Bacillus clausii</i>	YP_176339
<i>Streptococcus</i> phage <i>NZ131.2</i>	YP_002285775
<i>Listeria</i> <i>welshimeri</i>	YP_849416
<i>Streptococcus agalactiae</i>	ZP_00782383



**Figure 8.** RinA homologues control expression of the morphogenetic clusters of bacteriophages from other Gram-positive bacteria. Expression of  $\beta$ -lactamase was assayed in RN4220-derivative strains containing plasmids carrying *terS*-*blaZ* fusions and expressing or lacking the cognate *rinA* gene from phages found in *S. pyogenes* (pJP543 and pJP544), *E. faecalis* (pJP535 and pJP537) and *L. lactis* (pJP564 and pJP565). Samples were normalized for total cell mass.

## DISCUSSION

*Staphylococcus aureus* can adapt to different niches and hosts, producing a wide spectrum of acute and chronic diseases. Most of the variation between different *S. aureus* strains is due to the presence of MGE such as plasmids, prophages, pathogenicity islands, transposons and insertion sequences (24–27). These MGEs are clearly relevant in the pathogenic process of this bacterium, since many virulence factors are encoded on such mobile elements. In particular, bacteriophages play an important role in the pathogenicity of *S. aureus* either by carrying accessory virulence factors such as PVL (encoded by the *luk-PV* operon), staphylokinase (encoded by *sak*), enterotoxin A (encoded by *sea*) and exfoliative toxin A (encoded

by *eta*) or by interrupting chromosomal virulence genes such as those for  $\beta$ -hemolysin (*hlb*) and lipase (*geh*) upon insertion [reviewed in (1)]. Phages are also the primary vehicle of lateral gene transfer between *S. aureus* strains, providing the species with the potential for broad genetic variation. It has been reported recently that phages increase the genome plasticity of *S. aureus* during infection, facilitating the adaptation of the pathogen to various host conditions (28). Additionally, certain staphylococcal phages carry out a highly specialized example of phage-mediated HGT, the mobilization of SaPIs. SaPI replication and high frequency transduction is closely linked to the phage lytic cycle, which induces SaPI replication and provides the proteins required for SaPI packaging and transfer [for review see (10)]. Despite the clear importance of staphylococcal phages to *S. aureus* pathogenesis, and the large number of sequenced staphylococcal phage genomes now available in public genome databases, the number of studies characterizing the biology of these relevant elements is limited.

To ensure tight control of genes required in the later stages of infection, bacteriophages have evolved a variety of mechanisms involving synthesis of phage-encoded control factors during the early stages of infection. In this work, we have identified the activator of phage late transcription and localized the promoter responsible for the expression of the morphogenetic genes in several representatives from a large family of related *S. aureus* phages. This promoter is active only during the late phase of the lytic cycle and requires the phage-encoded RinA protein for activity. The late promoter is located upstream of the morphogenetic cluster and controls the synthesis of a single large operon encoding the proteins responsible for assembly of phage particles, DNA packaging and host cell lysis. Expression of this gene cluster is essential not only for propagation of the phage, but also for transfer of phage-borne toxin genes, generalized transduction and the horizontal transmission of other MGEs that exploit the phage for their own transfer, such as SaPIs. As would be expected for an activator of late transcription, preliminary analysis of  $\phi 11$ -encoded genes after SOS induction, using tiling arrays, indicate that *rinA* is expressed earlier than *terS*, in a transcription unit containing genes involved in phage replication (data not shown).

Activation of late transcription by positive regulatory proteins is a strategy employed by a variety of phages with dsDNA genomes. Enterobacteriophage P2 and related phages encode a small zinc-binding protein, Ogr, that binds to conserved inverted repeats centered at about  $-55$  relative to the transcription initiation sites of P2 late promoters (29–32). Protein–protein interaction between Ogr and the  $\alpha$  subunit of the host RNA polymerase is required to stimulate P2 late gene transcription (33). Activation of phage T4 late promoters requires two phage-encoded proteins that function as core RNA polymerase subunits. T4 gp55 functions as a sigma factor that allows recognition of the 8 bp late promoter consensus motif, while gp33 is a coactivator for high level late transcription (34,35). T4 late transcription also requires a unique transcriptional activator, gp45, which is a sliding

clamp that interacts with gp55, gp33 and T4 DNA polymerase to couple transcription to phage DNA replication (36). Phage P1 late transcription requires the phage-encoded Lpa protein, which binds to a conserved sequence centered at  $-22$ , as well as host-encoded coactivator, SspA (37). The phage Mu late activator protein, C, binds to a region of Mu late promoters from  $-30$  to  $-55$  and stimulates both binding of RNA polymerase and subsequent promoter release (38–40). Much less is known about the mechanism of transcription activation in the dsDNA phages of Gram-positive bacteria. The classic example is *Bacillus subtilis* phage SP01, which employs successive phage-encoded sigma factors to direct the host RNA polymerase to phage middle and late promoters (41) and also requires a T4 gp45-like protein for late transcription (42). The regulatory protein p4 from *B. subtilis* phage  $\phi 29$  binds between  $-58$  and  $-104$  upstream of the late promoter transcription start site and activates through contact with the  $\alpha$  subunit of RNA polymerase (43,44). An activator of late gene transcription (Alt) has also been identified in the *L. lactis* phage TP901-1 (45). Alt binds to a promoter region located upstream of the *terS* gene, recognizing a series of repeats  $-76$  to  $-32$  relative to the transcription start site (46). A common feature of all of these mechanisms is the recognition of sequences within or just upstream of the RNA polymerase binding site by a protein involved in activation of late transcription. RinA, in contrast, appears to require, for binding, sequences located more than 155 bp upstream of the *terS* transcription start site. Since essentially the same  $+1$  was independently mapped by RACE for both  $\phi 11$  and  $80\alpha$ , and the  $80\alpha$  RNA was treated to eliminate molecules with 5' monophosphate termini, we have high confidence that this site corresponds to the actual start of transcription and not an end generated by processing. The large distance between the transcription start and sequences needed for RinA binding therefore suggests a mechanism for transcription activation that is more complex than a direct interaction between RinA and an adjacent RNA polymerase.

RinA was initially reported as an activator of the  $\phi 11$  *int* gene (11). In earlier work, Ye *et al.* (23) identified the putative promoter region for the  $\phi 11$  *int* gene and observed that the DNA sequence of the region between the translation start sites of the *int* and *xis* genes, which are transcribed divergently, was conserved between phages L54a and phage  $\phi 11$ , suggesting that this conserved region could contain the potential regulatory sites for expression of the *int* and *xis* genes. Using a reporter plasmid containing the gene *xy/E* under control of this intervening sequence, including the  $\phi 11$  *int* promoter, different cloned regions of the  $\phi 11$  genome were screened for activation of expression. It was found that regulatory proteins RinA and RinB were both required to activate expression of the  $\phi 11$  *int* gene, while RinA alone was capable of activating L54a *int* gene transcription (11). Surprisingly, our results with the effects of *rinA* and *rinB* mutants do not confirm this initial characterization, suggesting that control of *int* expression is more complicated than predicted. Furthermore, RinA from phage L54a is even more divergent from  $\phi 11$  than the RinA of  $\phi 69$ , sharing

only 25% amino acid identity. Since we have demonstrated that the RinA proteins from  $\phi$ 11 and  $\phi$ 69 recognize and bind to different specific DNA regulatory sequences, it is difficult to explain how the  $\phi$ 11 and L54a proteins could bind to a conserved DNA sequence between *int* and *xis* to activate  $\phi$ 11 *int* expression.

RinA homologues can be found in bacteriophages infecting a variety of Gram-positive bacteria. We tested several of these and found that all were transcriptional activators that controlled expression of *terS*, the first gene of the morphogenetic cluster, in their respective phages. Thus, we propose that this family of proteins represents a conserved strategy for regulating the packaging and transfer of many bacteriophages in Gram-positive bacteria. Interestingly, an NCBI Conserved Domain Database search indicates that the RinA family of phage proteins also shows weak similarity to another large family of transcriptional regulators, ArpU. ArpU was described as a regulator of cellular muramidase-2 of *Enterococcus hirae* (47). Although initially reported as a chromosomally encoded gene, ArpU appears to have been cloned from a prophage. ArpU homologues are also present in a variety of different Gram-positive bacteriophages, and, like RinA, are localized upstream of the first gene of the morphogenetic cluster of the phage. This suggests that the ArpU-related proteins have a similar role in activating bacteriophage late gene expression, although this has not been tested directly. The C-terminal half of RinA also shows limited similarity to a portion of several sigma factors corresponding to conserved region 4. The significance of this is unclear. This region of sigma is involved in recognition of the  $-35$  element of promoters (48). While it seems quite unlikely that RinA is functioning as a sigma factor, this may indicate that the C-terminal part of the protein contains the DNA recognition determinants. On the other hand, sigma region 4 is also a target for a number of transcription factors (49) so this could be a protein–protein interaction domain. Further investigation of the structure and function of RinA is clearly needed to determine its mechanism of action in activation of transcription.

Horizontal transfer of DNA can occur in bacteria by transformation, conjugation and transduction. In *S. aureus*, there is little evidence that transformation occurs. In addition, conjugative plasmids are not widespread and conjugative transposons are even less common. In contrast, many of the known *S. aureus* bacteriophages are generalized transducing phages that can package *S. aureus* chromosomal DNA as well as MGEs, and transfer them to other *S. aureus* strains (12). Transduction is likely the predominant mechanism of HGT in *S. aureus*. Since transfer of the different MGEs requires exploitation of the phage mechanisms involved in virion assembly and DNA packaging, characterization of the phage proteins controlling these processes are important in understanding how MGEs are transferred. In this work we have identified and characterized a key regulator of genes involved in phage assembly and packaging. This activator, RinA, is not only present in most of the

*S. aureus* phages, but also in phages infecting a wide variety of other Gram-positive bacteria. Deletion of this activator resulted not only in elimination of the phage titre, but also significantly reduced transfer of SaPI-encoded virulence factors, and is predicted to show a similar reduction in transmission of plasmid-encoded virulence factors. Thus, we have identified an important mechanism involved in phage-mediated HGT, which plays a key role in bacterial evolution and pathogenesis.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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