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Deposited on: 20 June 2013
RinA controls phage-mediated packaging and transfer of virulence genes in Gram-positive bacteria

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Received October 25, 2010; Revised February 18, 2011; Accepted March 4, 2011

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...
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 bacteriophage 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 φ11 and 80z, which serve as helpers for some of the well-studied SaPIs, as well as in the clinically relevant phage φSLT, which encodes the PVL toxin. Phage φ11 mobilizes SaPIbov1 while 80z induces several SaPIs, including SaPI1, SaPI2, SaPIn1, SaPIbov1 and SaPIbov2 (10). We demonstrate that the phage rιnA 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 OD₅₄₀ = 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 for Clinical Sequencing Laboratory.

Enzyme assays

β-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. β-Lactamase units are defined as (Vmax)/OD₆₅₀.

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 triplicates. The relative transcriptional levels within distinct experiments were determined by using the 2^(-ΔΔCT) method. The results show the average ± 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 XbaI and BamHI, and cloned into plasmid pGEX-4 T-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 Comassie 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 (EMSAs) 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 80α infection were pooled during a time course of 80α infection and concentrated to 12 μl 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 (Epicentre 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× reaction buffer (provided by the manufacturer), 1 mM GTP (including 1 μCi of α-32P 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 80α 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× SSC, 0.1% SDS and twice at 42°C for 10 min in 0.1× 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 80α 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 80α 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 NruI-digested pBR322 and individual clones were sequenced.

**RESULTS**

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

The virion proteins of 80α and 80α, 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 80α 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 80α prophage by constructing in-frame deletions. We also introduced SaPIbov1 containing a tetracycline marker inserted in the tst locus (SaPIbov1 tsr::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,
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 φ11 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 80α (Table 1), which was expected because both rinA (Supplementary Figure S1) and the entire morphogenetic gene cluster of 80α and φ11 (Figure 1) are essentially identical. In order to determine the generality of this mechanism, we also constructed a rinA mutant of phage φSLT, which encodes a RinA homolog that is only 23% identical to that of φ11 (Supplementary Figure S1), although similar to RinA proteins encoded by other staphylococcal phages (data not shown). We used a detoxified derivative of phage φSLT (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_φ11), and pJP838 (pCN51-rinA_φSLT), carrying the rinA genes from φ11 and φSLT, 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 φ11 and 80α 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 φ11 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 φ11 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 terminator small subunit gene (terS; ORF29 φ11), the first gene in the morphogenetic gene cluster. To determine the transcription start site
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, minilysates were prepared, the DNA was separated on agarose and blotted with a phase- or SaPIbov1-specific probe, as indicated. The upper band is ‘bulk’ DNA, including chromosomal, phase and replicating SaPI DNA; the lower band is SaPI linear monomers released from phase heads.

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

Table 1. Effect of phage mutations on phage titre and SaPI transfer

<table>
<thead>
<tr>
<th>Donor strain</th>
<th>φ</th>
<th>Plasmid</th>
<th>SaPI</th>
<th>Phage titre</th>
<th>Transduction titre</th>
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*The means of results from three independent experiments are presented. Variation was within ±5% in all cases.

*aPhu/ml of induced culture, using RN4220 (or pCN51-complemented RN4200) as recipient.

*aTransductants/ml of induced culture, using RN4220 as recipient.
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 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 rinA$_{\phi 69}$ 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 rinA$_{\phi 11}$, 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 factor-independent terminator is indicated in Supplementary Figure S2.

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. RN451 or its derivative rinA mutant strain JP4028 containing plasmid pJP742 (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/OD$_{650}$).
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 80α 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 80α 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 80α, 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
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 φSa3ms, a related temperate siphovirus that carries a gene for staphylococcal enterotoxin SEA just upstream of the lysin 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 φ11, with the corresponding region from φ69, generating strain JP7188. We also introduced SaPIbov1 tsst::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 φ11 will not bind to the new RinA<sub>φ69</sub>-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 φ69 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 φ11<sub>int</sub> gene (11). To investigate this further, we analyzed int expression in φ11<sub>wt</sub> 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 φ11, φ11 ΔrinA and φ11 Δint (as a control), using primers hybridizing to the regions flanking the φ11 attB.
site. As expected, excision of the φ11 rinA mutant was similar to wt, while no excision was observed in the φ11 Δint 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 φ11 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 φ11 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 plasmids 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 β-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>
<thead>
<tr>
<th>Species</th>
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<tr>
<td>Lactobacillus casei</td>
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<tr>
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Figure 8. RinA homologues control expression of the morphogenetic clusters of bacteriophages from other Gram-positive bacteria. Expression of β-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 β-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 φ11-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 z 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 φ29 binds between −58 and −104 upstream of the late promoter transcription start site and activates through contact with the α 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 φ11 and 80z, and the 80z 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 φ11 int gene (11). In earlier work, Ye et al. (23) identified the putative promoter region for the φ11 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 φ11, 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/LE under control of this intervening sequence, including the φ11 int promoter, different cloned regions of the φ11 genome were screened for activation of expression. It was found that regulatory proteins RinA and RinB were both required to activate expression of the φ11int 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 φ11 than the RinA of φ69, sharing
only 25% amino acid identity. Since we have demonstrated that the RinA proteins from φ11 and φ69 recognize and bind to different specific DNA regulatory sequences, it is difficult to explain how the φ11 and L54a proteins could bind to a conserved DNA sequence between int and xis to activate φ11 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 morphogenic 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.

FUNDING

Consolider-Ingenio CSD2009-00006, BIO2005-08399-C02-02, BIO2008-05284-C02-02 and BIO2008-00642-E/C from the Ministerio de Ciencia e Innovacio´n (MICINN), and grants from the Cardenal Herrera-CEU University (PRCEU-UCH39/10 and Copernicus-Banco Santander program), from the Conselleria de Agricultura, Pesca i Alimentacio´ (CAPIA) and from the Generalitat Valenciana (ACOMP07/258) to J.R.P., grants [BFU2008-01078] from the MICINN and [2009SGR1106] from the Generalitat de Catalunya to J.B., NIH grants [R21 AI067654 and R56 AI018137] and a grant-in-aid from the A.D. Williams Trust and the Baruch Foundation Trust to G.E.C., a VCU Graduate School Thesis and Dissertation Award to M.D.H., and NIH grant [R01AI022159-23A2] to R.P.N. Funding for open access charge: Consolider-Ingenio CSD2009-00006, BIO2005-08399-C02-02, BIO2008-05284-C02-02 and BIO2008-00642-E/C from the Ministerio de Ciencia e Innovacio´n (MICINN), Spain.

Conflict of interest statement. None declared.

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