Biochemical insights into the function of phage G1 gp67 in *Staphylococcus aureus*  
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Bacteriophage (phage) are among the most diverse and abundant life forms on Earth. Studies have recently used phage diversity to identify novel antimicrobial peptides and proteins. We showed that one such phage protein, *Staphylococcus aureus* (*Sau*) phage G1 gp67, inhibits cell growth in *Sau* by an unusual mechanism. Gp67 binds to the host RNA polymerase (RNAP) through an interaction with the promoter specificity σ subunit, but unlike many other σ-binding phage proteins, gp67 does not disrupt transcription at most promoters. Rather, gp67 prevents binding of another RNAP domain, the α-C-terminal domain, to upstream A/T-rich elements required for robust transcription at rRNA promoters. Here, we discuss additional biochemical insights on gp67, how phage promoters escape the inhibitory function of gp67, and methodological advancements that were foundational to our work.

Introduction

*Staphylococcus aureus* (*Sau*) is a gram-positive bacterium of significant clinical importance.¹ Differences in transcriptional profiles drive the switch from commensal to pathogenic growth profiles, and these changes have been studied extensively using genetic and high-throughput approaches.¹⁻⁵ However, relatively few studies have examined transcription in *Sau* using mechanistic, biochemical and structural tools.⁶⁻⁸ Studies on bacteriophage (phage) have been fundamental to our understanding of molecular biology in prokaryotes. Early studies using phage elucidated many of the mechanisms of transcription and replication, in addition to understanding how phage modulate these critical processes to favor viral production over host cell functions.⁹⁻¹⁸ Due to the rise of antibiotic resistance in *Sau*, recent studies have examined the use of *Sau* specific phage as a platform to design novel therapeutics, or even for direct use as therapeutic agents.¹⁹,²⁰ These studies have largely used high-throughput techniques to identify proteins or peptides with antimicrobial effects, but have failed to perform the structural and mechanistic analyses required to evaluate whether the host targets would be accessible by traditional drug design processes.¹⁹,²⁰

Our recent work examined the mechanism of one such phage protein, *Sau* phage G1 gp67.²¹ Gp67 was identified as a putative RNA polymerase (RNAP) inhibitor and subsequently shown to bind to *Sau*, but not *Eco*, RNAP.¹⁹ Dehbi et al.¹⁹ showed that gp67 interacts with domain 4 of the housekeeping sigma factor (σ₄) in *Sau*. Biochemical analysis suggested that gp67 blocked −35 recognition, a mechanism of RNAP inhibition known to be exploited by other phage proteins.¹⁹,²² Dehbi et al.¹⁹ used well-characterized *Eco* proteins and promoters in their biochemical studies.

We sought to understand the mechanism through which gp67 blocked RNAP activity and cell growth by solving its structure in complex with σ₄. However, the structure showed that gp67 did not appear to block promoter DNA recognition or the interaction between core RNAP and σ₄ that is required for promoter-specific RNAP activity. Our subsequent biochemical analysis showed...
that a native Sau transcription system was absolutely required to the inhibitory effect of gp67. We showed, using in vitro biochemical and in vivo approaches, that gp67 does not block -35 recognition but rather modulates the binding that gp67 does not block −35 recognition but rather modulates the binding site. gp67 from the 2.0 Å co-crystal structure is shown in (A) and σ₄ from the co-crystal structure is shown in orange as a cartoon representation. The most highly conserved surface residues in gp67 map to the interaction between gp67 and σ₄.²¹

Our studies showed that gp67 inhibits Sau RNAP and subsequently Sau growth by an unusual mechanism. The structural data, in combination with the development of a native Sau in vitro transcription system that used Sau RNAP and Sau promoters were critical to our ability to examine the mechanism of inhibition by gp67. Additionally, our work on this phage protein allowed us to identify novel promoters in Sau and evaluate rRNA transcription, and its regulation, in this pathogenic organism. Further biochemical and structural work in gram positive organisms should bear in mind the importance of using native components in vitro experiments, despite the relative ease of using more well-developed model organisms such as Eco.

In this article, we will expand on our work on gp67 and provide additional biochemical detail on this protein and its interactions, discuss how phage promoters likely escape gp67 function, and extend our discussion on the methodological advancements required to study gp67.

Structure of apo-gp67

After solving the X-ray crystal structure of the complex between Sau σ₄ and gp67, we attempted to solve the structure of gp67 alone. While the protein expresses well and is easily purified to homogeneity, extensive screening for crystallization conditions did not yield any hits.

To determine whether gp67 was well folded, we performed limited proteolysis on gp67 alone and in complex with full-length σ₄ and σ₄. While gp67 resisted proteolysis to relatively high protease concentration in the presence of its binding partner (either σ₄ or σ₄), gp67 was readily cleaved even at low protease concentration in the absence of σ (Fig. 1). Gp67 alone in solution is likely poorly structured and undergoes a significant conformational change upon binding to RNAP. Along with the extended network of interactions between gp67 and σ₄, this may explain the tight binding between the proteins and the fact that we found evidence for gp67 bound to Sau RNAP even at promoters that were not directly inhibited.²¹ Experimental evaluation of binding kinetics could confirm this hypothesis.

Conservation of gp67 in Phage Genomes

BLAST searches using the sequence of phage G1 gp67 found five homologs with an E-score of < 0.1. All of the putative homologs are found in phage that infect gram-positive organisms, including phage specific to Bacillus, Enterococcus and Listeria. Gp67 also has a homolog in the Sau phage Twort. We showed that expression of the Twort homolog of gp67 in Sau cells also inhibits logarithmic cell growth, arguing for functional conservation between these two proteins.²¹

Using the program Consurf,²⁴ we used the structure of phage G1 gp67, and an alignment of the gp67 homologs, to map conservation onto the crystal structure. All but one of the universally conserved
residues in gp67 are hydrophobic amino acids in the core of the protein, evidence of structural conservation between gp67 homologs. Additional regions of conservation map to the binding surface with the conserved region of $\sigma^A_4$, arguing that all gp67 homologs bind similarly to the host RNAP (Fig. 2).

Our work shows the gp67 alone is sufficient to block normal Sau growth by blocking robust rRNA transcription. However, other phage proteins that bind to host RNAP are known to also interact with additional phage proteins to specifically recruit RNAP to phage promoters. Performing pull-downs with tagged gp67 or RNAP in phage infected Sau cells could easily identify any gp67-binding partners of phage or host origin. We find it likely that gp67 expressing phages, which do not encode their own RNAP, have complex coordination of transcription throughout the phage life cycle. Subsequent studies on G1, phage Twort and other phages may reveal these mechanisms of transcriptional regulation.

**Phage Promoters that Control Expression of gp67**

The G1 phage ORF67 (encoding gp67) is located downstream of a perfect consensus −10/−35 promoter and we therefore expect

| Phage | Promoter Locations |
|-------|--------------------|
| Staphylococcus phages G1/K | accggagttaatattttatatccacTTGACAtttatatgtaaGgtATAATTatattta 95% |
| Lactobacillus Phage Lb338-1 | agaaacttgaataagaagataaaaaaaactcTTGACAgcttttattgtagaataaatagcatg 80% |
| Listeria Phage A511 | cagagctgaggataatcctcagtttctttaTTGACTttatttagtaatagtagTATACTaaagttta 60% |
| Enterococcus Phage phiEF24C | agatatatctaggctttttgcgtgtactaTTGACTttatttagatattttgaatTATATAATgattta 60% |

**Figure 3.** Sequence of promoters that drive gp67 expression in phage genomes. (A) −35 and −10 elements are highlighted in red, as are the extended −10 elements (TGN immediately upstream of the 10 element). The region expected to act as a putative UP-element is highlighted in green and the percent A/T richness of this region is shown to the right of the sequences. (B) gp67 does not inhibit Sau RNAP at the phage G1 gp67 promoter. RNAP holoenzyme (50 nM) was incubated with gp67 at the indicated concentration and promoter DNA (50 nM) and reactions were initiated with 200 nM CTP/GTP/UTP and 50 nM ATP with 0.1 μL α-PATP. After 10 min reactions were stopped with 2x formamide buffer, boiled, electrophoresed on a 12% Urea-PAGE gel and visualized by autoradioaphy. Results from three independent experiments were quantified, normalized to the signal in the absence of gp67, and averaged, and are expressed as a mean in the graph below each lane (error bars represent one standard deviation above and below the mean).
that it is one of the phage genes that is initially expressed upon injection of the dsDNA phage genome into the host cell. Gp67 is then translated and modifies the host RNAP, which affects the transcription of host genes, including the important rrn promoters.21 However, phage promoters that contain UP-elements could also be inhibited by gp67. Furthermore, many G1 promoters contain clear evidence for an UP-element (up to 100% A/T; region upstream of the −35 element) that modulates transcription initiation.22 The initial biochemical work on gp67 suggested that it blocked −35 recognition.22 In contrast, our results show that gp67 does not affect −35 binding, as this would lead to inhibition at all promoters that require this interaction.21 The initial studies were performed using Eco RNAP and phage promoters.22 We attempted to reproduce these results but were unable to do so21; additional experiments using Eco RNAP and host promoters also produced inconclusive results. Even using Sau RNAP on well-studied Eco RNAP promoters did not produce consistent evidence for inhibition by gp67 (Hochschild A, personal communication). Sau is an A/T-rich gram-positive organism, and the DNA topology at promoters and kinetics of transcription initiation may not be the same as in Eco. It was only when we examined Sau promoters, and in particular the Sau rrn promoters, that we saw clearly reproducible effects of gp67 on RNAP output.21 Using RNA-seq to identify additional gp67-sensitive promoters that could be tested in vitro was also critical to forming our mechanistic hypotheses.21 Differences have also been described in transcription initiation between E. coli and Bacillus subtilis29,30 and recent work has examined the basis for the differences in promoter stability and initiation between E. coli and the thermophilic bacteria.31,32 Based on these results and our work, we suggesting using fully native transcription systems whenever possible.

Gp67 Effect on Promoter Stability

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The Use of Heparin as a DNA Competitor in in vitro Transcription Assays

To test RNAP activity in a single-round assay, or to isolate kinetic steps in the transcription cycle, competitor must be used to prevent RNAP re-binding to the promoter element. For decades, heparin has been used as a non-specific competitor to block RNAP/DNA interactions.\textsuperscript{34,38} More recent work has used large excesses of tight-binding dsDNA promoter elements identified by in vitro selection for RNAP binding using\textsuperscript{9} (FullCon promoter).\textsuperscript{39} In addition to competing away RNAP that has dissociated from the test promoter element after elongation or due to RNAP disassociation, heparin has been documented to actively destroy RNAP/promoter complexes.\textsuperscript{35,40}

In Sau, we found that using heparin in our in vitro transcription system severely decreased transcriptional output. In fact, at most Sau promoters, the presence of heparin in the reaction led to little or no detectable transcription. However, when we used the FullCon promoter construct as competitor,\textsuperscript{9} at 20-fold excess, we were able to detect RNA products, measure single round transcription levels, and determine open-promoter complex lifetimes (Supp. Materials and Methods; Fig. 4). In many organisms, the use of heparin in in vitro transcription assays may be ill advised, and the use of more gentle methods to block RNAP re-binding, such as using competing dsDNA promoter fragments, may be preferable.

Concluding Remarks

Gp67 illustrates the diversity of biological functions utilized by phage. This small protein has no sequence or structural homology to any known protein or fold. Our initial hypothesis, based on previously published work,\textsuperscript{19,20} was that gp67 inhibited RNAP using a mechanism previously ascribed to phage-encoded anti-\(\sigma\) factors. However, our structural and biochemical work quickly challenged these assumptions. In the end, gp67 functions by binding to \(\sigma\) but modulating the binding state of the RNAP \(\alpha\)-CTD to upstream promoter elements.\textsuperscript{21}

The phage T4 encodes a protein that ADP-ribosylates the \(\alpha\)-CTD of Eco RNAP at R265, the residue responsible for the interaction with the minor groove of UP-elements (Gourse R, personal communication). This effectively eliminates the ability of the \(\alpha\)-CTD to interact productively with UP-elements, blocking robust rRNA transcription. Gp67 acts by a similar mechanism but does not covalently modify RNAP.\textsuperscript{35} Rather, it forms a stable ternary complex with RNAP through its interaction with \(\sigma\). Based on our structural modeling, gp67 likely only blocks the proximal \(\alpha\)-CTD/UP-element interaction, which appears to be sufficient for RNAP inhibition at \(rrn\) promoters.\textsuperscript{21} The molecular detail of the interaction between \(\sigma\) the Sau \(\alpha\)-CTD and gp67 in the context of promoter DNA is of great interest. Crystallization of ternary complexes containing DNA may reveal the details of these interactions.

Studying phage biology has contributed to our understanding of many central mechanisms of transcription and DNA replication in prokaryotic cells.\textsuperscript{13,17,41-47} Relatively little work has been done on transcription in Sau using biochemical and structural tools. Our research clearly shows that the use of common in vitro methods to evaluate transcriptional differences in Sau upon the expression of a transcription factor with single-nucleotide resolution (Submitted). We consider it likely that species specific differences in promoter sequences and transcription regulation, which have been described between \(B\text{sub}\) and \(E\text{co}\),\textsuperscript{48} are present in other species as well. The tools developed in our work will be of great use in the continued examination of the basic mechanisms of transcription in Sau, and for further evaluation of the differences in transcription regulation between \(E\text{co}\) and other bacteria of clinical significance.\textsuperscript{49}

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/bacteriophage/article/24767

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