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# Inhibition of Transcription in *Staphylococcus aureus* by a Primary Sigma Factor-Binding Polypeptide from Phage G1▽

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**The primary sigma factor of *Staphylococcus aureus*, $\sigma^{SA}$, regulates the transcription of many genes, including several essential genes, in this bacterium via specific recognition of exponential growth phase promoters. In this study, we report the existence of a novel staphylococcal phage G1-derived growth inhibitory polypeptide, referred to as G1ORF67, that interacts with $\sigma^{SA}$ both in vivo and in vitro and regulates its activity. Delineation of the minimal domain of $\sigma^{SA}$ that is required for its interaction with G1ORF67 as amino acids 294 to 360 near the carboxy terminus suggests that the G1 phage-encoded anti-σ factor may occlude the −35 element recognition domain of $\sigma^{SA}$. As would be predicted by this hypothesis, the G1ORF67 polypeptide abolished both RNA polymerase core-dependent binding of $\sigma^{SA}$ to DNA and $\sigma^{SA}$-dependent transcription in vitro. While G1ORF67 profoundly inhibits transcription when expressed in *S. aureus* cells in mode of action studies, our finding that G1ORF67 was unable to inhibit transcription when expressed in *Escherichia coli* concurs with its inability to inhibit transcription by the *E. coli* holoenzyme in vitro. These features demonstrate the selectivity of G1ORF67 for *S. aureus* RNA polymerase. We predict that G1ORF67 is one of the central polypeptides in the phage G1 strategy to appropriate host RNA polymerase and redirect it to phage reproduction.**

Transcription initiation is a critical regulatory step in cell metabolism, and components of the transcription machinery are validated targets for antibacterial drug discovery, as witnessed with the successful use of rifampin over the last 4 decades (5, 37, 44). In bacteria, the RNA polymerase (RNAP) core enzyme associates with one of several sigma factors to form the RNAP holoenzyme, thereby directing efficient transcription from specific promoters (4, 14, 26, 31). During exponential growth, $\sigma^{70}$ of *Escherichia coli* and its orthologs from other bacteria are the primary σ factors, responsible for transcribing most growth-related and housekeeping genes. All primary σ factor orthologs share four distinct regions ($\sigma_1$ to $\sigma_4$) of highly conserved amino acid sequence with similar functions (4, 31). Regions $\sigma_2$ and $\sigma_4$ are involved in the direct contact with −10 and −35 promoter elements, respectively (4, 31).

Alternate σ factors recognize different promoter sequences and thereby direct the core enzyme to transcribe specific genes in response to changes in environmental conditions (14, 26, 41). The global gene expression profile is primarily exerted at the level of competition between various σ factors for the core enzyme (26). In *Staphylococcus aureus*, four different σ factors have been reported to date: the primary σ factor $\sigma^{SA}$ that directs the transcription of housekeeping genes during exponential growth phase (6) and three alternate σ factors, namely SigB, which modulates the expression of stress response genes (7, 19); SigH, which is required for transcribing competence genes (30); and a novel extracytoplasmic function sigma factor named SigS that appears to be an important component of the stress and pathogenic responses (41).

The diversity of σ factors, their abundance in the cell, and their relative affinities for the RNAP core enzyme provide a sophisticated mechanism dictating the coordinated spatiotemporal expression of genes in response to specific environmental conditions (14, 26). Given this key role, σ factors are targeted by a wide range of transcriptional regulators, such as activators (43), repressors (28), competitors (26), small RNAs (45), and anti-σ factors (13). The binding of an anti-σ factor to its cognate σ factor leads to specific inhibition of transcription of the genes that are regulated by that particular σ factor (13). As an example, the bacteriophage T4-encoded anti-σ factor AsiA (32, 36) exerts a critical shift in the phage infective cycle by redirecting transcription by host RNAP to phage middle promoters. Recent structural studies reveal that AsiA binding to *E. coli* $\sigma^{70}$ results in remodeling of domains that contact the −35 element of the promoter in conjunction with the T4 MotA protein while leaving the contacts between σ and the −10 region intact (4, 11, 22). Predicted orthologs of AsiA have been found in the genomes of other T4-like phages, as well as in genomes of some gram-negative bacteria, including *E. coli* and *Pseudomonas aeruginosa* (8, 15, 16, 18). This family of anti-$\sigma^{70}$ factors shares key amino acid residues known to be crucial for the binding of AsiA to *E. coli* $\sigma^{70}$ (36).

We recently reported the results from a phage genomics and functional genomics study based on protein-protein interaction between phage-carried growth inhibitory polypeptides and proteins of the bacterial host (23). In *Staphylococcus aureus*, such an approach identified a variety of host proteins that regulated key metabolic pathways, such as DNA replication and transcription. In most cases, these host proteins were shown to be essential for cell viability.

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In the present study, we extend our studies to the staphylococcal bacteriophage G1, a member of the *Myoviridae* phage family, like T4 (21), and characterize a phage-encoded growth inhibitory polypeptide, designated G1ORF67, as an anti-σ factor that binds tightly to the primary sigma factor of *S. aureus*. The G1ORF67 binding domain on $\sigma^{SA}$ is delineated, and the consequences of such interaction are assessed by functional assays both in vivo and in vitro.

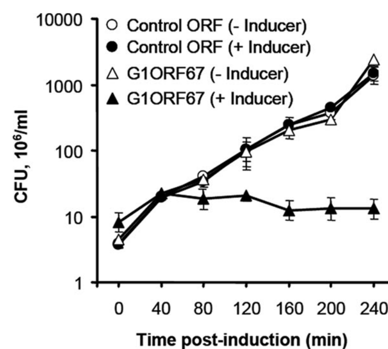


FIG. 1. Growth inhibition kinetics of *S. aureus* expressing G1ORF67. Clones of *S. aureus* RN4220 harboring either G1ORF67 or a control ORF (which has no impact on *S. aureus* growth) under the regulation of an arsenite-regulatable promoter were grown in tryptic soy broth supplemented with 30 μg/ml kanamycin with or without 5 μM $NaAsO_2$. At different time intervals, aliquots of the cultures were plated onto tryptic soy agar plates supplemented with kanamycin in order to determine the number of CFU. Results are expressed as the means ± standard deviations of the results for three independent clones.

## MATERIALS AND METHODS

**Reagents.** Restriction and modification enzymes were obtained from New England Biolabs. Lysostaphin, Na-arsenite, poly(dI-dC), streptomycin sulfate, phenylmethylsulfonyl fluoride, heart muscle kinase, silver nitrate, and glutathione agarose resin were purchased from Sigma. $Ni^{++}$-nitrilotriacetate agarose resin was purchased from Qiagen. Affi-gel resin and protein assay kit were from Bio-Rad. Protease inhibitor cocktail was obtained from Roche Diagnostics. RNasin pancreatic RNase inhibitor was obtained from Promega. The *E. coli* RNAP holoenzyme and core enzyme were purchased from Epicentre Technologies. Radioactive nucleotides and precursors were from GE Healthcare. Europium (Eu)-conjugated anti-$His_6$ antibody was purchased from CIS-Bio International; anti-influenza hemagglutinin antibody was from Babco. Allophycocyanin (APC)–anti-glutathione *S*-transferase (GST) antibody and -streptavidin conjugates were from Prozyme. Multiscreen GF/B plates were purchased from Millipore.

**Growth inhibitory property of the phage polypeptide.** The growth inhibitory property of G1ORF67 was characterized in a time-kill broth assay essentially as described previously (23). Briefly, ORF67 was amplified by PCR from phage G1 (21) by using a sense primer, 5′-CGGGATCCATGAAATTAAAGATTTTAGA-3′, in conjunction with the antisense primer 5′-CCCAAGCTTCTATTTACTAATTTTTTTCA-3′. The PCR product was digested with BamHI/HindIII and cloned into the unique BamHI/HindIII sites of expression vector pTM under the control of Na-arsenite (23). *S. aureus* RN4220 was used as the host strain for monitoring the growth inhibitory property of G1ORF67 in the time-kill assay.

**Identification of the bacterial target for G1ORF67 by affinity chromatography.** The G1ORF67 polypeptide (NCBI Entrez protein accession number YP_240941 [21]) was purified from *E. coli* BL21(DE3) as a $His_6$ fusion using $Ni^{++}$-chelate chromatography and cross-linked to Affi-gel 10 resin at protein/resin concentrations ranging from 0 to 7 mg/ml. Subsequent chromatographic steps with lysate from *S. aureus* RN4220 were performed as previously described (23).

**Validation of G1ORF67-$\sigma^{SA}$ interaction.** The interaction between the phage polypeptide and the bacterial protein was validated essentially as described previously for *S. aureus* DnaI and the 77ORF104 polypeptide (NCBI Entrez protein accession number NP_958646 [23]). For far-Western analysis, *S. aureus* $\sigma^{SA}$ was purified from *E. coli* BL21(DE3) as a fusion protein tagged at its N terminus with the heart muscle kinase phosphorylation site (17) and a $His_6$ tag. The recombinant protein was radiolabeled with [γ-$^{32}$P]ATP and heart muscle kinase and used as a probe with immobilized phage polypeptides. In the time-resolved fluorescence resonance energy transfer (TR-FRET) (27) assay, G1ORF67 was purified from *E. coli* BL21(DE3) as a GST fusion; its interaction with purified $His_6$-tagged $\sigma^{SA}$ was detected by using antitag antibodies conjugated to APC and Europium (Eu), respectively, as described previously (23). Yeast-two-hybrid analysis was performed using a Matchmaker two-hybrid system 3 according to the manufacturer's instructions (CLONTECH Laboratories).

**Overexpression and purification of *S. aureus* RNAP core enzyme.** The *S. aureus rpoA* gene encoding the α subunit of RNAP was PCR amplified from genomic DNA of *S. aureus* strain RN4220 using the sense oligonucleotide 5′-CGGGATCCATGATAGAAATCGAAAAACCTAGA-3′ and the antisense oligonucleotide 5′-ACGCGTCGACACTATCTTCTTTTCTTAATCCTAA-3′. The PCR product was digested with BamHI/SalI and cloned into pTM (23) as a C-terminal fusion with tandem affinity purification tags consisting of $His_6$ and the biotin acceptor domain (2) and used to transform *S. aureus* RN4220. Cells were grown in tryptic soy broth (TSB) with 30 μg/ml kanamycin to an optical density at 540 nm of 0.5 and induced with 10 μM Na-arsenite for 2 h at 37°C. Bacteria were harvested by centrifugation. The bacterial pellet from a 30-liter culture was resuspended in 400 ml HNG-1000 buffer (20 mM HEPES–KOH, pH 8.0, 1 M NaCl, and 10% glycerol) supplemented with 10 mM imidazole, protease inhibitor cocktail, 1 mM phenylmethylsulfonyl fluoride, and approximately 30,000 U of lysostaphin. The cell suspension was incubated at 37°C for 30 min, and cells were lysed by sonication. Nucleic acids were precipitated with 3% streptomycin sulfate for 20 min at 4°C. The RNAP core enzyme was purified essentially free of $\sigma^{SA}$ by $Ni^{++}$-nitrilotriacetic acid chromatography using wash buffers HNG-1000 and TGEN (10 mM Tris-HCl, pH 8.0, 5% glycerol, 150 mM NaCl, and 0.1 mM EDTA), each supplemented with 10 mM imidazole, and using as elution buffer TGEN with 200 mM imidazole. The identities of the purified subunits (α, β, and β′) were confirmed by mass spectrometry of tryptic digests (23; data not shown).

**In vitro transcription assays.** In vitro transcription assay reactions were similar to those described previously (6) and were performed with increasing concentrations (range, 0 to 500 nM) of $\sigma^{SA}$ and 25 nM of *E. coli* core enzyme in a total volume of 25 μl containing 40 mM Tris–acetate, pH 7.9; 100 mM NaCl; 5 mM $MgCl_2$; 1 mM dithiothreitol; 0.1 mg/ml bovine serum albumin (BSA); 0.5 mM of ATP, GTP, and CTP; 0.25 mM UTP; 5 μCi [α-$^{32}$P]UTP (3,000 Ci/mmol); 1 U RNasin; and 40 ng pB6 template DNA. Plasmid pB6 is a derivative of the previously described pZE21 vector harboring a cassette driven by the $\lambda P_L$ promoter, the kanamycin gene, and the ColE1 RNA1 gene (25). To investigate the effect of G1ORF67 on $\sigma^{SA}$-dependent transcription, the $\sigma^{SA}$-specific phage polypeptide G1ORF67 and the negative control polypeptide 77ORF104 (23) were purified as GST fusions and preincubated (final concentration, 2 μM) with $\sigma^{SA}$ for 10 min on ice prior to the addition of the other reagents. Reaction mixtures were incubated at 37°C for 15 min, stopped with formamide loading buffer, and electrophoresed on a denaturing gel.

Alternatively, the gel-based assay was converted to a miniaturized high-throughput trichloroacetic acid (TCA)-based assay as follows: $\sigma^{SA}$ (100 nM) was mixed with *S. aureus* core enzyme (50 nM) in a total volume of 25 μl containing 40 mM Tris–acetate, pH 7.9; 100 mM NaCl; 5 mM $MgCl_2$; 1 mM dithiothreitol; 0.1 mg/ml BSA; 150 μM each ATP, GTP, and CTP; 30 μM UTP; 100,000 cpm [α-$^{32}$P]UTP (3,000 Ci/mmol); 1 U RNasin; and 40 ng pB6 template DNA. The effect of G1ORF67 was monitored by including the purified phage polypeptide or a negative-control polypeptide (each at a final concentration of 10 μM) in the reaction mixture, followed by an incubation of 1 h at 37°C in a 96-well PCR plate. Samples were transferred to 96-well multiscreen plates and subjected to a 10% TCA precipitation step in the presence of 10 μg salmon sperm carrier DNA. The radiolabeled RNA product was counted by using a liquid scintillation counter (Trilux 1450 Microbeta; PerkinElmer).

**In vitro DNA-binding studies.** A TR-FRET assay for formation of RNAP-promoter oligonucleotide nucleoprotein complexes was developed as follows. The 5′ end of the sense strand of the −41 to −12 sequence of the $\lambda P_R$ promoter oligonucleotide (10) was biotinylated and annealed to its complementary strand for use as a probe. The assay was performed in a 24-μl volume containing 20 mM HEPES, pH 8.0, 100 mM KCl, 1 mM EDTA, 400 mM KF, 200 nM BSA, 3% glycerol, 50 nM biotin-tagged oligonucleotide probe, 32 nM His-tagged $\sigma^{SA}$, and 10 nM of *E. coli* core enzyme. The reaction mixture was incubated for 15 min at room temperature, and 6 μl of a mixture of Eu-conjugated anti-$His_6$ and APC-conjugated streptavidin was added to final concentrations of 3 and 15 nM, respectively. Samples were mixed, and 25 μl of the mixture was transferred to a black 96-well plate (Molecular Devices). After 45 min of incubation at room

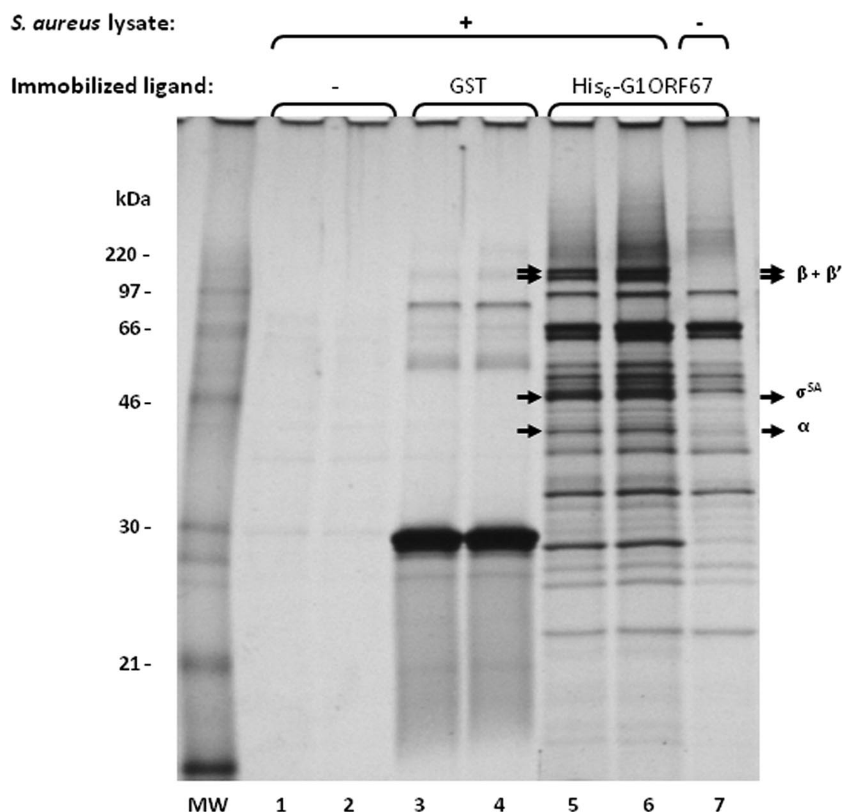


FIG. 2. G1ORF67 interacts with the RNAP holoenzyme of *S. aureus*. Results of SDS-PAGE of 1% SDS eluates from His-tagged G1ORF67 (lanes 5 to 7) or GST (lanes 3 and 4) in affinity chromatography. Experiments were performed in duplicate with a mock-immobilized resin and *S. aureus* lysate (lanes 1 and 2), with a resin containing immobilized GST with *S. aureus* lysate (lanes 3 and 4), and with a resin containing immobilized His-tagged G1ORF67 and *S. aureus* lysate (lanes 5 and 6). A resin containing immobilized His-tagged G1ORF67 with no input lysate (lane 7) served as a further control. The positions of migration of the β, β′, $\sigma^{SA}$, and α polypeptides are indicated to the right of the gel image. The masses, in kDa, of protein standards are indicated to the left of lane "MW." Eluates were separated by SDS-PAGE and visualized with silver nitrate. Specific polypeptide bands were excised from the gel and subjected to tryptic peptide mass determination by liquid chromatography and electrospray tandem mass spectrometry. +, present; −, absent.

temperature, the fluorescence signals (excitation, 340 nm; Eu emission, 612 nm; and APC emission, 665 nm) were measured using an Ultra plate reader (Tecan). The specificity of the interaction was monitored by including one of three non-biotinylated oligonucleotides (−35 sequence is underlined and in boldface): (i) parental $\lambda P_R$, 5′-ATGATA**TTGACT**TATTGAATAAAATTGGGT-3′; (ii) $\lambda P_L$, 5′-GATAGAGA**TTGACA**TCCCTATCAGTGATAGAGATACTGAGCACAT CAGC-3′; or (iii) Mut $\lambda P_R$, 5′-ATGATA**ACTTTG**TATTGAATAAAATTGGG T-3′ (as control, with scrambled −35 element). In competition analyses, an excess of untagged oligonucleotide was added to the reaction mixture prior to the addition of the Eu and APC conjugates. The effect of G1ORF67 on the DNA-binding activity of $\sigma^{SA}$ was monitored by including increasing amounts of the phage polypeptide (0 to 4 μM) in the reaction mixture prior to the addition of Eu and APC conjugates.

## RESULTS

**Characterization of phage G1ORF67 as an inhibitor of *S. aureus* growth.** We applied a functional genomics approach (23) to characterize staphylococcal bacteriophage G1 (21). Accordingly, a total of 214 open reading frames (ORFs) of at least 33 amino acids were predicted to be encoded by the phage genome (21). In dot screen-based assays (23), seven ORFs were shown to abolish bacterial growth when their expression was induced in *S. aureus* strain RN4220 (data not shown). One of these ORFs, termed G1ORF67, with a predicted molecular mass of 25 kDa, is the subject of the current study. In order to establish the kinetics of the growth inhibition mediated by G1ORF67, we induced its expression and monitored its effect on *S. aureus* viability, measured as CFU, over time. The results indicate that the phage polypeptide exerted a bacteriostatic effect upon its induction since, unlike the uninduced growth control, bacterial CFU remained constant from 40 min to 240 min following induction of G1ORF67 synthesis (Fig. 1). As a negative control, another phage polypeptide whose expression did not show inhibition of bacterial growth in a dot screen-based assay was also examined in an identical manner (Fig. 1). The observed growth inhibitory effect of G1ORF67 appeared to be specific to *S. aureus*, since its expression in *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* did not yield any growth inhibitory effect (data not shown).

**G1ORF67 binds directly to the primary sigma factor of *S. aureus*, $\sigma^{SA}$.** In an attempt to identify cellular proteins that mediated the growth inhibitory effect of G1ORF67, we expressed and purified G1ORF67 as a $His_6$ fusion protein from *E. coli* and used it as a column ligand in affinity chromatography studies. Whole-cell lysates from *S. aureus* were then loaded onto a $His_6$-G1ORF67 column and a GST control column. After extensive washes, bound proteins were eluted with 1%

sodium dodecyl sulfate (SDS), resolved by SDS-polyacrylamide gel electrophoresis (PAGE), and stained with silver nitrate (Fig. 2). The bands of interest were excised from the gel and subjected to trypsin digestion, and their identities were revealed by mass spectrometry as described previously (23). Accordingly, four polypeptides with apparent masses of 35 kDa, 45 kDa, and >100 kDa bound selectively to the $His_6$-G1ORF67 column (Fig. 2, lanes 5 and 6) and not to the GST column (Fig. 2, lane 3 and 4). As a further control, the four polypeptides were absent in eluates from a $His_6$-G1ORF67 column that had not received *S. aureus* lysate (Fig. 2, lane 7). Hence, the recovery of these polypeptides was dependent on both $His_6$-G1ORF67 and *S. aureus* lysate (Fig. 2). Mass spectrometry of tryptic digests of the polypeptides indicated that they are components of *S. aureus* RNAP holoenzyme, consisting of the $\alpha$ subunit (35-kDa band); the primary sigma factor, $\sigma^{SA}$ (45-kDa band); and the $\beta$ and $\beta'$ subunits (>100-kDa bands). Under the experimental conditions described here and for three independent G1ORF67 affinity chromatography experiments, no targets other than the RNAP holoenzyme were reproducibly identified.

To validate the interaction between G1ORF67 and subunit(s) of the *S. aureus* RNAP, we performed a series of in vitro and cell-based interaction assays. We focused initially on $\sigma^{SA}$ as a potential interacting protein, since the corresponding primary sigma factor of *E. coli*, $\sigma^{70}$, is known to be the target of the T4 phage-carried transcriptional regulator AsiA (32, 36). In protein affinity (far-Western) blotting, a concentration-dependent hybridization signal was detected between the immobilized G1ORF67 polypeptide and a radiolabeled $\sigma^{SA}$ probe (Fig. 3A), thereby confirming that the interaction between G1ORF67 and $\sigma^{SA}$, as initially detected by affinity chromatography, was direct. No interaction between the $\sigma^{SA}$ probe and immobilized control polypeptide 77ORF104 was detected (Fig. 3A). Like G1ORF67, 77ORF104 inhibits the growth of *S. aureus* when expressed intracellularly (23); however, 77ORF104 binds to a different *S. aureus* protein (DnaI) (23), and its sequence is unrelated to that of G1ORF67 (11% similarity at the amino acid level).

The interaction between G1ORF67 and $\sigma^{SA}$ was also confirmed by the TR-FRET assay (27), a solution-phase assay for monitoring protein-protein interactions (23). Accordingly, the 50% inhibitory concentration, at which 50% of the signal was inhibited, for the interaction between the phage polypeptide and $\sigma^{SA}$, as determined by competition with untagged G1ORF67, was estimated to be approximately 30 nM (Fig. 3B).

**The G1ORF67 polypeptide binds to a region of $\sigma^{SA}$ comprising conserved regions 4.1 and 4.2.** To delineate the minimal region of $\sigma^{SA}$ capable of binding to G1ORF67, we employed the yeast two-hybrid assay. Coexpression of G1ORF67 and full-length $\sigma^{SA}$ allowed growth of the recombinant yeast strain under selective conditions (Fig. 4A), regardless of whether the phage polypeptide or the bacterial protein was expressed as a fusion with the GAL4 transactivation or DNA-binding domain (Fig. 4A and data not shown). This finding indicated that under the conditions of the yeast two-hybrid assay, G1ORF67 and $\sigma^{SA}$ interact, in concurrence with the results from far-Western blotting and the TR-FRET assay presented above. Next, a series of $\sigma^{SA}$ truncation mutants were tested with full-length G1ORF67 as combinatorial pairs for

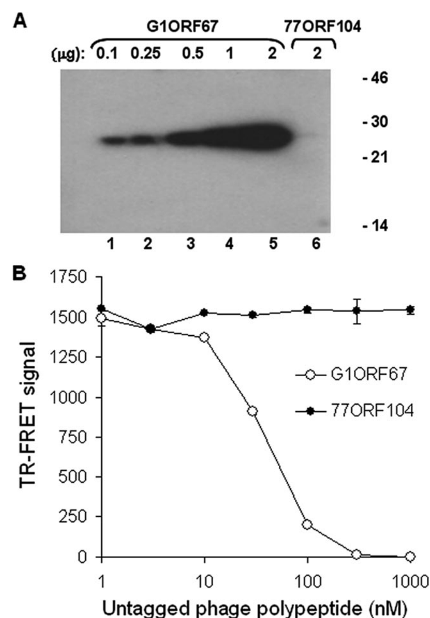


FIG. 3. Confirmation of the direct interaction between G1ORF67 and *S. aureus* $\sigma^{SA}$. (A) Far-Western analysis demonstrates the direct interaction of $\sigma^{SA}$ with G1ORF67. Increasing amounts of purified His-tagged G1ORF67 (100 ng to 2 µg, lanes 1 to 5) or 2 µg of 77ORF104 (lane 6) were separated by SDS-PAGE, immobilized onto a nitrocellulose membrane, and probed with $^{32}P$-labeled $\sigma^{SA}$. Protein standards (masses in kDa) are indicated on the right. (B) Dose-response study of the interaction between G1ORF67 and $\sigma^{SA}$ by TR-FRET as described in Materials and Methods. Untagged G1ORF67 or 77ORF104 polypeptides were used as competitors. Error bars show standard deviations.

their ability to confer growth on yeast on selective medium. A fragment of 67 amino acids (residues 294 to 360) near the C terminus of $\sigma^{SA}$ was sufficient for its binding to G1ORF67 (Fig. 4C). Interestingly, this region contains conserved regions 4.1 and 4.2, of which region 4.2 is involved in the direct contact with the $-35$ consensus element (9). Truncation of the N-terminal portion of this 67-amino-acid fragment, resulting in loss of amino acids 294 to 316 (and thereby most of region 4.1, which comprises amino acids 300 to 321 in $\sigma^{SA}$), led to the loss of G1ORF67 binding (Fig. 4B and C). Similarly, truncation of the C terminus of full-length $\sigma^{SA}$, resulting in the loss of amino acids 329 to 368 (and thereby practically all of region 4.2, which comprises amino acids 328 to 354 in $\sigma^{SA}$), resulted in an inability to support growth of the recombinant strain on selective medium (Fig. 4B and C). Hence, the two-hybrid system delineated residues 294 to 360 of $\sigma^{SA}$, containing regions 4.1 and 4.2, as the minimal region required for binding to G1ORF67.

**The RNAP core-dependent DNA-binding ability of $\sigma^{SA}$ is abolished by G1ORF67.** The finding that the $-35$ consensus element-binding region of $\sigma^{SA}$ was necessary and sufficient for interaction with G1ORF67 prompted us to test the hypothesis that core-dependent DNA-binding activity of $\sigma^{SA}$ would be impaired in the presence of G1ORF67. As a prelude to this experiment, we developed a solution-phase TR-FRET assay to monitor RNAP core-dependent DNA-binding activity of $\sigma^{SA}$. This assay used as a probe a biotinylated duplex oligonucleo-

FIG. 4. In vivo interaction of G1ORF67 with *S. aureus* $\sigma^{SA}$. (A) Yeast two-hybrid analysis. Constructs encoding full-length $\sigma^{SA}$ (amino acids 1 to 368) as a fusion with GAL4 activation domain (AD) and G1ORF67 as a fusion with GAL4 DNA-binding domain (DNA BD) were generated and used to transform the yeast strain. Cotransformants were plated on selective medium lacking tryptophan and leucine (TL−) or lacking tryptophan, histidine, adenine, and leucine (THAL−). (B) Schematic representation of domain organization of *S. aureus* $\sigma^{SA}$. (C) Mapping of the minimal domain of *S. aureus* $\sigma^{SA}$ that interacts with G1ORF67. Constructs encoding full-length $\sigma^{SA}$ (amino acids 1 to 368) or its truncated derivatives were cloned as fusions with the GAL4 activation domain and used in combination with full-length G1ORF67/GAL4 DNA-binding domain in transformations. Cotransformants were plated on selective medium, and the growth or absence of growth of the yeast strain was interpreted as an interaction (+) or absence of an interaction (−), respectively, between $\sigma^{SA}$ and G1ORF67.

tide representing a truncated version of $\lambda P_R$ consisting of the sequence from −41 to −12 (10). This probe thus contains the −35 consensus element but lacks the −10 sequence. The biotinylated, truncated $\lambda P_R$ oligonucleotide was incubated with His-tagged $\sigma^{SA}$ and RNAP core enzyme; Eu-conjugated anti-$His_6$ antibodies and APC-conjugated streptavidin were then added. In the absence of G1ORF67, a strong TR-FRET signal from the APC fluorescence acceptor was detected upon excitation of the Eu fluorescence donor (Fig. 5A, 0 nM competitor). Since in this assay RNAP core was strictly required for binding of $\sigma^{SA}$ to the $\lambda P_R$ oligonucleotide (data not shown), this result indicated that $\sigma^{SA}$ possesses core-dependent DNA-binding activity on the $\lambda P_R$ oligonucleotide containing the consensus −35 element.

In competition analyses, an excess of nonbiotinylated oligonucleotide was added to the reaction mixture prior to the addition of the Eu and APC conjugates. The parental $\lambda P_R$ and $\lambda P_L$ oligonucleotides were found to compete efficiently in a concentration-dependent manner for the interaction between RNAP holoenzyme and biotinylated $\lambda P_R$ promoter fragment (Fig. 5A). As would be predicted for a specific interaction between the RNAP holoenzyme and a duplex DNA bearing both −35 and −10 consensus sequences, a mutated version of the $\lambda P_R$ oligonucleotide containing a scrambled −35 consensus sequence ($A_{-35}$CTTTG in place of $T_{-35}$TGACT) failed to compete for binding (Fig. 5A).

The effect of G1ORF67 on the core-dependent DNA-binding activity of $\sigma^{SA}$ was monitored by including increasing amounts of the purified phage polypeptide in the RNAP holoenzyme-promoter oligonucleotide mixture prior to the addi-

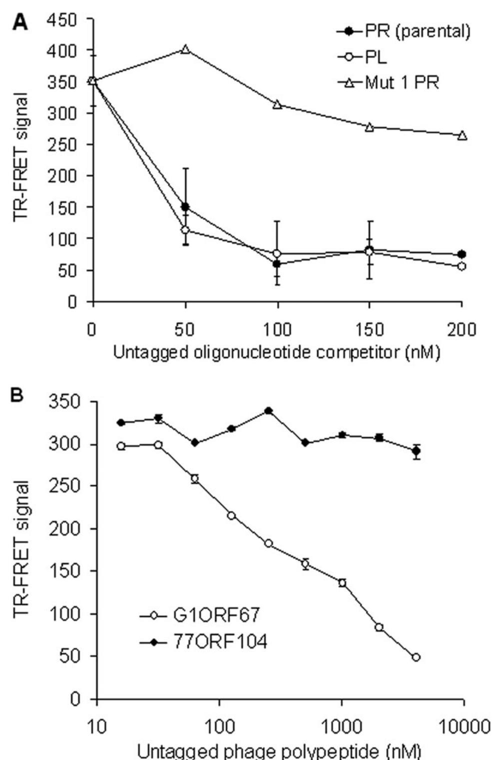


FIG. 5. Core-dependent DNA binding of $\sigma^{SA}$ and inhibition by G1ORF67. (A) Competition for core-$\sigma^{SA}$-$\lambda P_R$ oligonucleotide binding as monitored by TR-FRET. Nonbiotinylated $\lambda P_R$ and $\lambda P_L$ oligonucleotides and a nonbiotinylated mutant version of the $\lambda P_R$ oligonucleotide containing a scrambled −35 regulatory sequence element (Mut 1 PR) were used as competitors. (B) Dose-dependent inhibition of the binding of core-$\sigma^{SA}$ to $\lambda P_R$ by G1ORF67 as determined by TR-FRET. An unrelated growth-inhibitory phage polypeptide, 77ORF104, was used as negative control. Error bars show standard deviations.

tion of Eu and APC conjugates. G1ORF67 specifically inhibited the ability of core-$\sigma^{SA}$ to bind to its cognate promoter DNA in a dose-dependent manner (Fig. 5B), whereas the purified control polypeptide 77ORF104 had no impact on core-dependent DNA binding by $\sigma^{SA}$.

**The G1ORF67 polypeptide inhibits $\sigma^{SA}$-dependent transcription in vitro.** The findings that G1ORF67 both interacted with $\sigma^{SA}$, as shown above, and specifically inhibited transcription in *S. aureus* cells (23) prompted us to develop a $\sigma^{SA}$-dependent in vitro functional assay to directly test the effect of purified G1ORF67 polypeptide on transcription. Initially, in vitro transcription assays comprised the *E. coli* RNAP core enzyme complemented with purified $\sigma^{SA}$ and a DNA template containing $\sigma^{SA}$-regulated promoters; radiolabeled transcription products were monitored by gel electrophoresis and autoradiography. Transcription from the $\lambda P_L$ promoter was stimulated by $\sigma^{SA}$ in a dose-dependent manner (Fig. 6A). The RNA1 gene from ColE1 was also transcribed by $\sigma^{SA}$ in a dose-dependent manner (data not shown), a finding consistent with previous work of Deora and Misra (6). The addition of purified G1ORF67 polypeptide to the reaction mixture abolished $\sigma^{SA}$-dependent transcription (Fig. 6B; compare lane 2 to lane 1). To determine whether the inhibition observed was specific to G1ORF67, we tested the effect of purified phage polypeptide 77ORF104 on transcription and found that transcription was unaffected in the presence of the unrelated phage polypeptide (Fig. 6B, lane 3). Interestingly, G1ORF67 lacked inhibitory activity in transcription assays with the *E. coli* RNAP holoenzyme (Fig. 6C). This outcome is consistent with the inability of G1ORF67 to interact with *E. coli* $\sigma^{70}$ in the yeast two-hybrid system (data not shown). Taken together, these results demonstrate selectivity of G1ORF67 for the primary sigma factor of *S. aureus*.

We sought to determine whether G1ORF67 also had an inhibitory effect on $\sigma^{SA}$-dependent transcription in the context of *S. aureus* RNAP. To this end, the core enzyme of *S. aureus* was copurified to near homogeneity (Fig. 6D). Transcription products from the *S. aureus* RNAP core, from the holoenzyme, and from the holoenzyme in the presence of phage polypeptides were subjected to TCA precipitation and quantitated by liquid scintillation counting. The *S. aureus* RNAP core enzyme, which had been purified under conditions of high stringency to remove $\sigma^{SA}$, had only weak activity (Fig. 6E, bar 1). In contrast, when purified $\sigma^{SA}$ was added back to the core enzyme, transcription was stimulated 10-fold (Fig. 6E, bar 2). As was seen above with $\sigma^{SA}$-dependent transcription by the *E. coli* RNAP core, the ability of $\sigma^{SA}$ to stimulate transcription by the *S. aureus* core was abolished with G1ORF67 (Fig. 6E, bar 3) but not with 77ORF104 (Fig. 6E, bar 4). Under these conditions, the 50% inhibitory concentration of G1ORF67 for the in vitro transcription reaction was approximately 0.2 $\mu$M (data not shown). These results clearly indicated that G1ORF67 inhibits $\sigma^{SA}$-dependent transcription regardless of whether $\sigma^{SA}$ is in association with the RNAP core enzyme of *S. aureus* or *E. coli*. Together, the findings illustrate that G1ORF67 interacts with $\sigma^{SA}$ and acts as a transcription inhibitor at $\sigma^{SA}$-regulated promoters.

## DISCUSSION

In the present study, we characterized the mechanism by which G1ORF67, a staphylococcal bacteriophage polypeptide, inhibits the growth of *S. aureus*: it binds to the primary sigma factor of *S. aureus* and inhibits transcription at $\sigma^{SA}$-dependent promoters. These results extend our previous observation of specific inhibition of transcription in *S. aureus* cells upon expression of G1ORF67 in that they suggest a mechanism by which the phage polypeptide may inhibit *S. aureus* transcription at −35 consensus promoters. This activity may be central to host shutoff early in the G1 phage infective cycle. Given both its direct interaction with $\sigma^{SA}$ and its ability to inhibit $\sigma^{SA}$ function, we conclude that G1ORF67 is a phage-encoded anti-$\sigma$ factor directed against $\sigma^{SA}$ of *S. aureus*.

AsiA of phage T4 is a prototypic anti-$\sigma$ factor with specificity for $\sigma^{70}$, the primary sigma factor of *E. coli* (32). Recent models propose that AsiA first binds to free $\sigma^{70}$ and that this complex then binds to the RNAP core to form AsiA-bound holoenzyme (1, 12, 22, 24, 38, 42). The AsiA binding determinants of $\sigma^{70}$ have been mapped to the C-terminal region; they consist of conserved regions 4.1 and 4.2 (29, 39, 40), of which region 4.2 is primarily responsible for the binding of $\sigma^{70}$ to the canonical −35 promoter element (4, 24, 29). Binding of AsiA to $\sigma^{70}$ has been demonstrated to preclude the binding of $\sigma^{70}$ to the canonical −35 promoter element, thereby inhibiting transcrip-

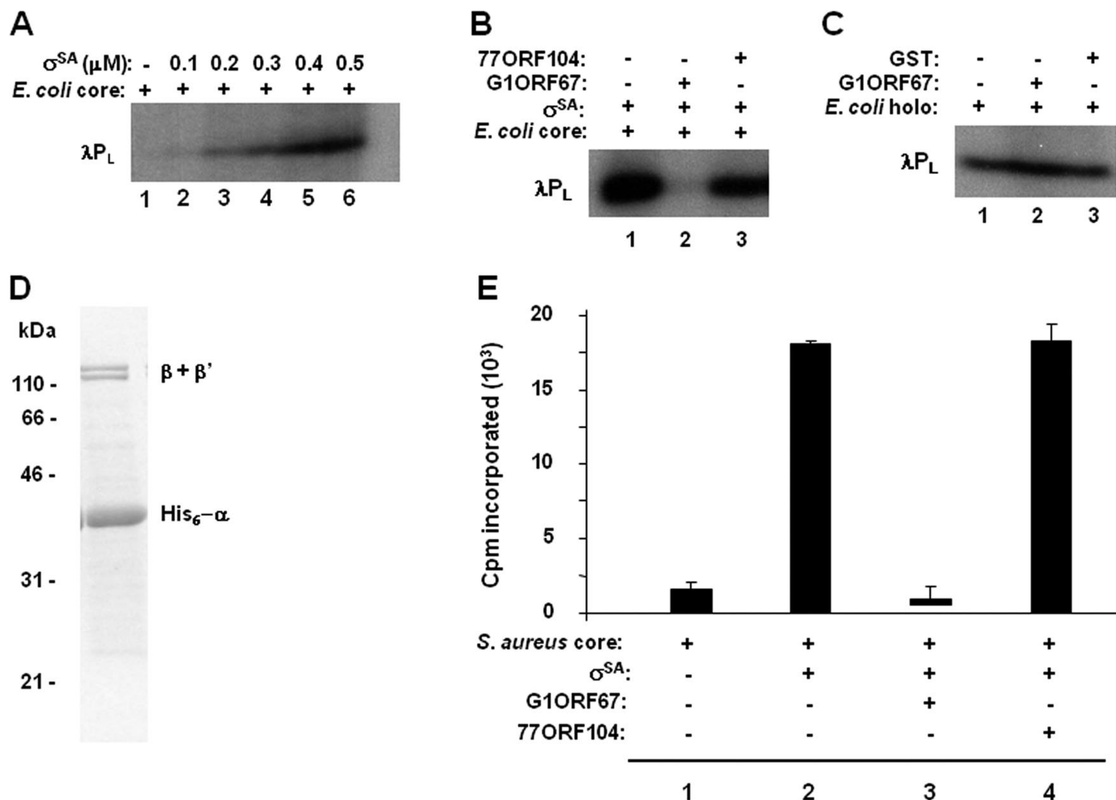


FIG. 6. Inhibition of $\sigma^{SA}$-dependent transcription in vitro by G1ORF67. (A) Dose-dependent stimulation of the transcriptional activity of *E. coli* core enzyme from $\lambda P_L$ promoter. The amount of $\sigma^{SA}$ is indicated above each lane. The core enzyme was used at 25 nM. Transcription products were separated on a denaturing polyacrylamide gel and visualized by autoradiography. (B) Specific inhibition of $\sigma^{SA}$-dependent transcription from $\lambda P_L$ promoter by G1ORF67. *E. coli* core enzyme and $\sigma^{SA}$ were used at 25 nM and 500 nM, respectively. Where present, G1ORF67 and the negative-control phage polypeptide (77ORF104) were added to 2 μM (final concentration). (C) G1ORF67 does not inhibit in vitro transcription from the *E. coli* RNAP holoenzyme (*E. coli* holo). *E. coli* holoenzyme was used at 25 nM. Where present, G1ORF67 and the negative-control polypeptide (GST) were added to 2 μM (final concentration). (D) SDS-PAGE analysis of the endogenous RNAP core enzyme purified from *S. aureus* RN4220. Proteins were resolved by SDS-PAGE and stained with Coomassie blue. Protein standards (masses in kDa) are indicated to the left of the gel image. $His_6$-α and β + β′ indicate the migration positions of the histidine-tagged α subunit and of untagged β and β′ subunits of *S. aureus* RNAP as determined by tryptic fingerprinting and mass spectrometry. (E) Inhibition of $\sigma^{SA}$-dependent transcriptional activity of *S. aureus* core enzyme by G1ORF67 as monitored by TCA precipitation and liquid scintillation counting. The concentrations of *S. aureus* core enzyme and $\sigma^{SA}$ were 50 nM and 100 nM, respectively. Where present, G1ORF67 and the negative-control phage polypeptide (77ORF104) were added to 10 μM (final concentration). Error bars show standard deviations. +, present; −, absent.

tion of cellular genes that require an authentic −35 element (12, 36, 42). Similarly to AsiA, Rsd was shown to bind to region 4.2 and to inhibit transcription from $\sigma^{70}$ promoters (15, 40); however, in contrast to AsiA, Rsd prevents the binding of $\sigma^{70}$ to the core (35, 46). Likewise, region 4 of the flagellar-specific sigma factor $\sigma^{28}$ of *Salmonella enterica* serovar Typhimurium is required for binding the anti-sigma factor FlgM (20).

G1ORF67 is unrelated to AsiA (21% similarity at the amino acid level) and only marginally similar to Rsd (20% identity and 33% similarity), suggesting that these anti-sigma factors may inhibit transcription at −35 consensus promoters by different mechanisms. Alternatively, despite the significant level of amino acid sequence homology between region 4 of $\sigma^{70}$ and $\sigma^{SA}$ (43% identity and 62% similarity), anti-sigma factors may require different interactions for transcription inhibition or sigma appropriation (11). Using the yeast two-hybrid system under experimental conditions that validated the interaction between G1ORF67 and $\sigma^{SA}$, we were unable to detect AsiA-$\sigma^{SA}$ and G1ORF67-$\sigma^{70}$ interactions (data not shown). There are at least two possible mechanisms that could explain the observed inhibitory effect of G1ORF67 on $\sigma^{SA}$, as demonstrated in functional assays. The first possibility is that the phage polypeptide interacts with both free and RNAP core-bound $\sigma^{SA}$, thereby preventing its binding to the −35 promoter sequence and ultimately inhibiting transcription of genes requiring an authentic −35 consensus element. Alternatively, G1ORF67 may prevent the binding of $\sigma^{SA}$ to the RNAP core enzyme, thereby inactivating both $\sigma^{SA}$ and the core. However, our finding that in affinity chromatography, the α, β, and β′ subunits were coeluted along with $\sigma^{SA}$ (Fig. 2) supports the formation of a ternary complex consisting of G1ORF67-$\sigma^{SA}$-RNAP core, as was found for AsiA-$\sigma^{70}$-core (38). This finding argues against a mechanism by which G1ORF67 prevents $\sigma^{SA}$ from binding to the core enzyme. That the G1ORF67 binding site was mapped to a region containing regions 4.1 and 4.2 of $\sigma^{SA}$ supports this possibility and predicts that in the putative ternary complex, the surface on $\sigma^{SA}$ required for binding to the −35 recognition element would be occluded by G1ORF67.

Although our results from $\sigma^{SA}$-DNA-binding assays strongly support the notion that inhibition of $\sigma^{SA}$-DNA binding by G1ORF67 is due to interference with the required −35 contact region, they did not address whether this interference is direct (amino acid residues of $\sigma^{SA}$ required for its direct contact with the −35 consensus element are also involved in the interaction with G1ORF67) or indirect (through conformational change rendering regions 4.1 and 4.2 of $\sigma^{SA}$ inaccessible for binding to the −35 consensus element).

While the expression of G1ORF67 in growing *S. aureus* cells results in rapid and profound inhibition of host transcription (23), one of the outstanding questions raised by this study is the functional implication of G1ORF67-$\sigma^{SA}$ interaction for phage biology during the infection. That is, how is the genome of phage G1 transcribed if $\sigma^{SA}$ activity is impaired? Additionally, which phage G1 genes govern the expression of early versus middle or late functions and does G1ORF67 play a role in this process? In the context of phage T4, AsiA protein cooperates with T4 MotA to coactivate transcription from T4 middle promoters (3, 33, 34). Based on amino acid sequence homology, we did not find a MotA homologue in the genome of phage G1. This is perhaps not surprising given the absence of sequence homology between G1ORF67 and T4 AsiA. We predict that the binding of G1ORF67 to $\sigma^{SA}$ and the resulting inhibition of host $\sigma^{SA}$-dependent RNAP activity trigger a shift in the transcriptional profile of phage genes during infection. The importance of such a shift in gene expression would be to promote the expression of genes involved in phage maturation and release. G1ORF67 would thus appear to be one of the central polypeptides in phage G1's strategy to appropriate host RNAP and redirect it to phage reproduction.

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