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Contribution of teg49 Small RNA in the 5′ Upstream Transcriptional Region of *sarA* to Virulence in *Staphylococcus aureus*

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High-throughput RNA sequencing technology has found the 5′ untranslated region of *sarA* to contain two putative small RNAs (sRNAs), designated teg49 and teg48. Northern blot analysis disclosed that teg49 and teg48 were detectable within the P3-P1 and P1 *sarA* promoter regions, respectively. Focusing on teg49, we found that this sRNA, consisting of 196 nucleotides, is transcribed in the same direction as the *sarA* P3 transcript. The expression of both P3 and teg49 transcripts is dependent on *sigB* and *cshA*, which encodes a DEAD box RNA helicase. Within the sRNA teg49, there are two putative hairpin-loop structures, HP1 and HP2. Transversion mutation of the HP1 loop produced a smaller amount of *sarA* P3 and P2 transcripts and SarA protein than the corresponding HP1 stem and the HP2 stem and loop mutations, leading to lower RNAII transcription and derepression of *aur* transcription. The HP1 loop mutant also exhibited less biofilm formation than the parental and complemented strains. Complementation with shuttle plasmid pEPSA5 carrying teg49 was able to reestablish *sarA* P3 and P2 transcription and augment RNAII expression in the HP1 loop mutant. We thus conclude that teg49, embedded within the extended promoter regions of *sarA*, is modulated by *sigB* and *cshA* and plays an important *trans*-acting role in modulating the transcription and ensuing expression of *sarA*.

Staphylococcus aureus, an opportunistic pathogen, is a major cause of life-threatening infections in humans. The pathogenicity of *S. aureus* is shaped by a variety of extracellular and cell wall-associated proteins that are expressed in a growth phase-dependent fashion (1, 2). The expression of these virulence factors is controlled by a complex network of global regulatory elements, including *agr* and the staphylococcal accessory regulator (SarA) of *S. aureus*. The *sarA* locus encodes a DNA binding protein, SarA, that functions as a repressor or an activator by binding to conserved AT-rich DNA motifs (ATTTTAT) in the promoter regions of target genes in *agr*-dependent and *agr*-independent manners (3, 4).

The *sarA* locus in *S. aureus* has an open reading frame (ORF) of 372 bp with a predicted molecular mass of 14,718 Da and a deduced pI of 8.52 (5). Notably, the 5′ untranslated region (UTR) of the *sarA* locus spans 860 bp with three promoters (6), encompassing three distinct but overlapping transcripts of 0.58 (P1), 0.84 (P3), and 1.15 (P2) kb, all encoding the SarA protein. The expression of these three transcripts is known to be growth phase dependent, with P1 and P2 expressed during the exponential phase and P3 expressed postexponentially (6), and is thought to be required for the complete restoration of SarA function (6). Because of the unusual length of the *sarA* promoter region, it was hypothesized that the 5′ UTR may possess elements (e.g., small RNAs [sRNAs]) that are involved in optimal expression of the *sarA* gene.

S. aureus expresses a large number of sRNAs, many of which do not possess well-described functions (7, 8, 9, 10, 11). Recent studies have demonstrated that sRNAs of *S. aureus* are known to play a pivotal role in a variety of regulatory processes by base pairing with target mRNAs and by modulating protein activity (12, 13, 14, 15). For instance, sRNA RsaE pairs with the Shine-Dalgarno sequence of mRNAs of *opp-3B*/*opp-3A* (amino acid and peptide transporter) and *sucC* (succinyl-coenzyme A synthase) and prevents the formation of a ribosomal initiation complex. It has been suggested that RsaE may coordinate the downregulation of central metabolism when carbon sources become limited (12). ArtR, a toxin-regulating sRNA, is involved in virulence regulation by activating alpha-toxin expression. ArtR also promotes degradation of *sarT* mRNA by RNase III and arrests the translation of SarT by direct binding to the 5′ UTR of the *sarT* mRNA (13). SprD, a sRNA expressed from *S. aureus* pathogenicity islands (PIs), represses translation initiation by base pairing with *sbi* mRNA, leading to impaired adaptive and innate host immune responses (14). Morrison et al. (15) showed that SSR42, which is expressed predominantly during stationary phase, regulates the expression of several virulence factors, including protein A, capsule, α-hemolysin, and Panton-Valentine leukocidin toxin. SSR42 is also involved in resistance to human polymorphonuclear leukocyte killing and pathogenesis in a murine model of *S. aureus* infection (15).

Here we report the presence of an sRNA designated teg49 in the *sarA* promoter region located within the P3-P1 promoter region. Analyses by primer extension and 3′ rapid amplification of cDNA ends (RACE) revealed that teg49 is 196 nucleotides (nt) in length and located at nt 667081 to 666886 of the reference *S. aureus* N315 genome. The teg49 transcript and the *sarA* P3 transcript were diminished in cells lacking *sigB* or the DEAD box RNA helicase gene *cshA* but restored in the complemented mutants. There are two putative hairpin structures, designated HP1 and HP2, within the teg49 sRNA. Replacement of the 7 nt in the HP1 loop of teg49 in the chromosome led to truncated transcripts from the P2

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TABLE 1 Strains used in this study

S. aureus strain	Genotype and/or characteristic	Source or reference
RN4220	Mutagenized strain 8325-4 that accepts foreign DNA	17
SH1000	Functional *rsbU* derivative of 8325-4 *rsbU*$^+$	19
Newman	Isolated from a human infection in 1952	41
ALC6094	Strain Newman transduced with IPTG[b]-inducible T7 polymerase; Tet^r	42
ALC7201	ALC6094 Δ*cshA*::Kan^r	This study
ALC7252	ALC7201 Δ*cshA*::*cshA*	This study
ALC7286	SH1000 *sarA* deletion	This study
ALC7288	SH1000 HP1 stem mutation of teg49	This study
ALC7289	SH1000 HP1 loop mutation of teg49 (ATTGCGC→CGCTATA)[a]	This study
ALC7290	SH1000 HP2 loop mutation of teg49 (GTCGATT→TATCGGC)[a]	This study
ALC7291	SH1000 HP2 stem mutation of teg49	This study
ALC7869	Tn*551*::*sigB* in ALC6094	This study
ALC7870	ALC7869 containing pSK236::*sigB*	This study
ALC7909	ALC7289 containing empty vector pEPSA5	This study
ALC7911	ALC7289 containing pEPSA5::teg49	This study

[a] Nucleotides replaced in chromosomal DNA.
[b] IPTG, isopropyl-β-D-thiogalactopyranoside.

TABLE 2 Primers used in primer extension and 3′ RACE-PCR

Primer	Sequence
PS-1	CATTTTTAGTGATAAAATTTTG
PS-2	GGTAAATTATAAAAAATGCTG
PS-3	GTTTTATAAACACTTTTTTG
PS-4	GCAAAATTATGACTAACATATC
PA-11	CTAAAGAGATTCTTTGTTATAGC
PA-12	CAGCATTTTTTATAATTTACC
PA-13	CAAAAAAGTGTTTATAAAAC
PA-14	GATATGTTAGTCATAATTTTGC

and/or P3 promoters, resulting in reduced *sarA* expression, *agr* expression, and biofilm formation, analogous to what has been found in a *sarA* mutant.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. The bacterial strains used in this study are listed in Table 1. *S. aureus* strain Newman and its derivatives ALC6094, SH1000, and RN4220 were grown at 37°C in tryptic soy broth (TSB) or tryptic soy agar. *Escherichia coli* strains were grown in LB broth or LB agar. The following antibiotics were used when appropriate: erythromycin (5 μg/ml), chloramphenicol (10 μg/ml), tetracycline (2.5 μg/ml), and kanamycin (50 μg/ml) for *S. aureus* strains and ampicillin (100 μg/ml) and kanamycin (100 μg/ml) for *E. coli* strains.

DNA manipulations and transformation. Standard procedures for DNA manipulations were used for cloning (16). Restriction endonucleases and DNA-modifying enzymes were purchased from New England BioLabs. *E. coli* and *S. aureus* plasmids were isolated with the QIAprep Spin Miniprep kit (Qiagen). Transformations of *E. coli* and *S. aureus* cells were carried out with a MicroPulser (Bio-Rad). Recombinant plasmids obtained from *E. coli* were first transformed into *S. aureus* RN4220 for proper DNA methylation to reduce restriction barriers (17). The plasmids purified from RN4220 were then electroporated into *S. aureus* Newman derivative ALC6094 (18) or SH1000 (19).

Construction of mutants and complementation in *S. aureus*. Allelic exchange was carried out with the temperature-sensitive and Erm^r pMAD shuttle vector (20). The *cshA* mutant of strain ALC6094, a strain Newman derivative that contains a T7 polymerase gene at the *geh* site (18), was created with a kanamycin resistance cassette cloned into pMAD. Briefly, PCR was used to amplify a 1.3-kb fragment comprising a 0.74-kb fragment upstream and a 0.56-kb fragment downstream of *cshA* with genomic DNA as the template. The PCR fragment was cloned into pMAD, resulting in pMAD-Δ*cshA*. A SmaI-digested 0.9-kb fragment containing the kanamycin resistance cassette was inserted into pMAD-Δ*cshA* to create pMAD-Δ*cshA*::Kan^r. The recombinant shuttle vector was transformed into *S. aureus* ALC6094 (18). Transversion mutations of HP1 and HP2 loops and HP1 and HP2 stems in teg49 were introduced by PCR with primers with altered nucleotides, cloned into pMAD, and transformed into *S. aureus* SH1000. Allelic exchanges were performed as described previously (20). Selected mutants were subsequently complemented by reintroducing pMAD-*cshA* and pMAD-teg49 into the chromosome by homologous recombination as described previously (20). All of the mutant strains created were confirmed by PCR and sequencing.

Complementation of the HP1 loop mutant was also performed in *trans*. In this instance, we cloned teg49 immediately downstream of the xylose-inducible promoter in shuttle plasmid pEPSA5; this was followed by transformation into RN4220 and then into the HP1 loop mutant. Pilot Northern analysis indicated that teg49 was expressed in the HP1 loop mutant with recombinant plasmid pEPSA5::teg49 in the presence of 1% xylose. The pEPSA5 vector in the HP1 loop mutant served as the negative control.

RNA isolation, Northern blot analysis, and primer extension. Isolation of RNA and Northern blot hybridization were performed as previously described (18). Briefly, cells grown in TSB at 37°C were harvested at optical densities at 650 nm (OD_{650}s) of ~0.2, ~0.7, ~1.1, and ~1.7 with 18-mm borosilicate glass tubes in a Spectronic 20 (Spectronic Instrument), representing the early, mid-log, late log, and stationary phases, respectively. Pellets were resuspended in 1 ml TRIzol (Invitrogen) with 0.1-mm glass/zirconia beads, and RNA was extracted as described previously (18). Total RNA (10 μg) of each sample was electrophoresed through a 1.5% agarose–0.66 M formaldehyde gel in morpholinepropanesulfonic acid (MOPS) running buffer (20 mM MOPS, 10 mM sodium acetate, 2 mM EDTA, pH 7.0), transferred to a Hybond-N$^+$ membrane (Amersham), hybridized with gel-purified PCR fragments radiolabeled with [α-^{32}P]dCTP by the random-priming method (Ready-To-Go Labeling kit; GE) under high-stringency conditions (21), washed, and autoradiographed. Primer extension experiments were performed with Moloney murine leukemia virus reverse transcriptase according to the manufacturer's instructions (Ambion) and 5′ UTR-specific *sarA* oligonucleotide primers (Table 2).

Western blotting. Whole-cell extracts of *S. aureus* wild-type SH1000 or *sarA* mutants containing separate HP1 and HP2 stem and loop mutations grown to an OD_{650} of ~0.7 were prepared. The concentrations of total proteins from clear lysates were determined with the Pierce BCA Protein Assay kit (Thermo Scientific, IL) with bovine serum albumin as the standard. Western blotting and detection were performed as described previously (22).

Biofilm formation. Quantification of biofilm formation on abiotic surfaces was done as described elsewhere (23). Briefly, *S. aureus* grown overnight in TSB supplemented with 0.25% glucose (TSB-glucose) was diluted 1:40 in TSB-glucose. This cell suspension was used to inoculate sterile 96-well polystyrene microtiter plates (Iwaki Inc.) in triplicate. After 18 h of incubation at 37°C, wells were gently washed three times with 200 μl of sterile phosphate-buffered saline, air dried in an inverted position, and stained with 0.1% safranin for 30 s. Wells were rinsed again and solubilized with ethanol, and the absorbance at 550 nm was determined (FL800; BioTek Instruments). Each assay was repeated in five separate experiments. Colony morphology was studied on Congo red agar as previously described (24).

RNA-Seq. teg48 and teg49 were previously discovered and reanalyzed in the whole transcriptome of *S. aureus* N315 (10). Double-stranded

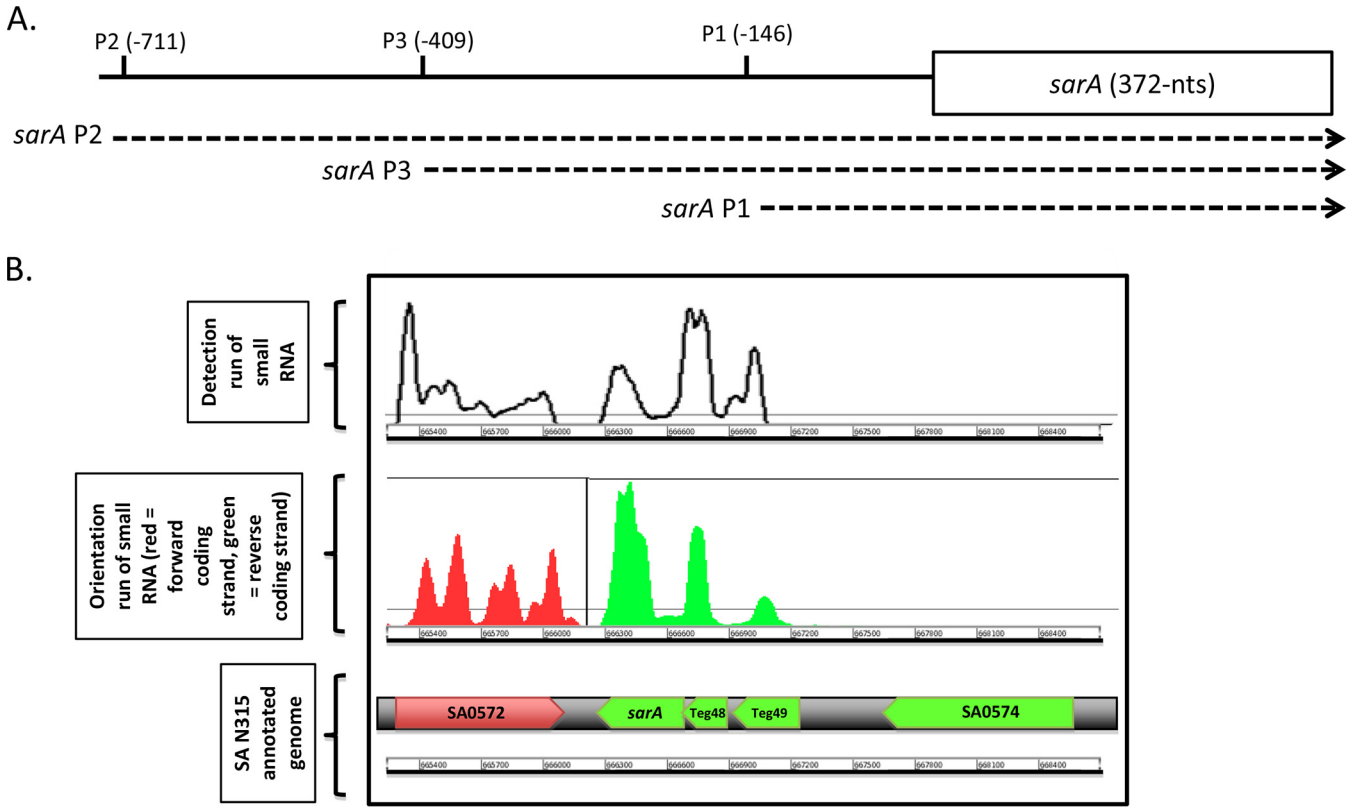

FIG 1 RNA-Seq analysis of the *sarA* locus. (A) Architecture of the *sarA* locus. Three promoters located between −710 and −146 upstream of the *sarA* translation start compose the topology of the *sarA* locus. (B) Schematic representation of the *sarA* region subjected to a transcriptomic study by RNA-Seq. An RNA-Seq detection run shows different transcript signals upstream of *sarA* (top). The orientation run (middle) shows the similar transcription orientation of the two smaller transcripts within the *sarA* locus and the transcription, in the opposite direction of *sarA*, of flanking gene *SA0572*, whereas the other flanking gene, *SA0574*, is not expressed under the conditions studied. The annotated genome is depicted at the bottom.

cDNAs were synthesized with random primers from DNase-treated RNA. cDNAs were fragmented by nebulization, ends were repaired, and fragments were ligated with Illumina genomic adapters. Size selection on agarose gel allowed the selection of inserts of approximately 30 to 150 bp that were used to construct the library by PCR amplification (see reference 10 for the detailed protocol). An sRNA orientation run was performed with total RNA purified with the MirVana isolation and MicrobExpress kits (Ambion) after 4 h of growth in rich medium. RNAs were prepared with a dir-mRNA-SEQ protocol. After end repair, RNAs were ligated with single-stranded sRNA adapters. After cDNA synthesis and PCR amplification for 15 cycles, the library was size selected on agarose gel to select inserts of 15 to 100 bp. Sequencing was performed with an Illumina GAII for 36 cycles. The reads obtained were mapped onto the annotated sequence of strain N315 (NC_002745) and analyzed with the Artemis genome viewer (25).

3′ RACE assay. *S. aureus* strain N315 was grown for 4 h in Mueller-Hinton broth from a 0.1 McFarland suspension obtained from a diluted overnight culture. Total RNA from a cell suspension treated with lysostaphin (50-μg/ml final concentration) was purified with the RNeasy kit (Qiagen). RNA quality and quantity were assessed with the Bioanalyzer (Agilent) and NanoDrop ND-8000.

As the region upstream of *sarA* contains two RNA species, the transcripts were separated and purified on a 10% acrylamide gel. Two fractions with sizes of <400 and >400 nt were purified. RNA fractions were polyadenylated with the MessageAmp Bacteria-Prokaryotic RNA amplification kit (Ambion). Poly(A) RNA fractions were immediately used for nonspecific synthesis of cDNA with the SMARTer RACE cDNA amplification kit (Clontech). For 3′ RACE-PCR, cDNA synthesis was performed with 3′-CDS primer A (1 μM final concentration) for 90 min at 42°C, followed by 10 min at 70°C. cDNAs were diluted in 20 μl Tricine-EDTA buffer before proceeding to specific 3′ RACE-PCR with Advantage 2 Polymerase Mix (Clontech) in accordance with the manufacturer's instructions in 1× Universal Primer Mix A and teg49 GSP2 primer at a 0.2 mM final concentration. Samples were amplified by 30 cycles of 30 s at 94°C, 30 s at 62°C, and 3 min at 72°C. PCR products were purified by QIAquick columns (Qiagen) and quantified. Finally, 3′ RACE-PCR products were sequenced with the BigDye Terminator Cycle Sequencing v.3.1 kit (Life Technologies) with a 3130 XL device (Applied Biosystems). Sequences were aligned with the N315 annotated genome sequence (RefSeq accession no. NC_002745) with Artemis software (25).

RESULTS

Identification of sRNA within the *sarA* locus by RNA-Seq. Beaume et al. (10) have previously described the use of RNA-Seq technology to obtain a representative map of the whole transcriptome of *S. aureus* strain N315. They identified 160 sRNA molecules in regions considered to be noncoding or intergenic. Some of the sRNAs are localized in biologically or clinically relevant regions, between key metabolic or virulence genes or within PIs, in strain N315 (10). Among these regions of interest, it was noticed that the *sarA* region contains two putative sRNAs. These data were reanalyzed, and we designated these sRNAs teg49 and teg48 (Fig. 1). The teg49 sRNA is located between the P3 and P1 promoters, while teg48 resides between the P1 promoter and the *sarA* trans-

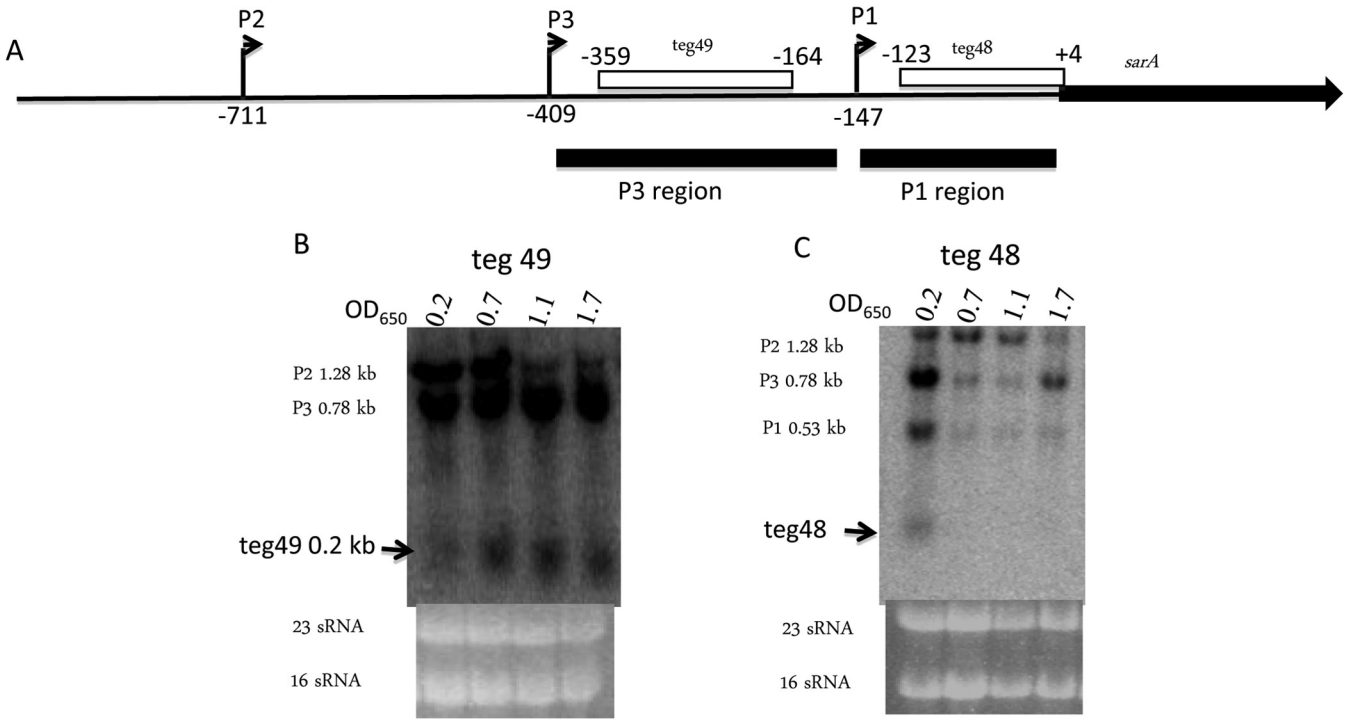

FIG 2 Two sRNAs, teg49 and teg48, are detectable in the 5′ UTR of the *sarA* gene. (A) Map of 5′ UTR and *sarA* ORF. Two sRNAs, teg49 and teg48, are indicated in the P3-P1 and P1 promoter regions, respectively. The transcription start sites of P2, P3, and P1 are marked at −711, −409, and −149, with +1 corresponding to the *sarA* translational start site, as indicated by bent arrows. The P3 and P1 regions (black bars) were used as probes for Northern blot assays. The locations of teg49 and teg48 in the 5′ UTR of *sarA* are indicated at −356 to −164 and −123 to +4 (empty bars), respectively, with +1 corresponding to the *sarA* translational start site. (B, C) Northern blot assay detection of sRNAs teg49 (B) and teg48 (C). Total RNA (10 μg) from *S. aureus* Newman derivative ALC6094 in various growth phases (see Materials and Methods) were separated in a 1.5% agarose gel, transferred to an H^+-bond membrane, and probed as described in Materials and Methods.

lation start site (Fig. 2A), as confirmed by reanalysis by RNA-Seq and Northern blot assay.

Northern blot analysis confirms the expression of two sRNAs, teg49 and teg48, within the 5′ UTR of *sarA*. To verify the existence of both teg49 and teg48, we performed Northern blot assays with radiolabeled P3 and P1 fragments (Fig. 2A). Total cellular RNA of strain ALC6094, a derivative of strain Newman (Table 1), was extracted at various time points after inoculation. A probe from the *sarA* P3 region (−409 to −147, where +1 is the *sarA* translational start site) hybridized with three distinct transcripts of 1.15, 0.8, and 0.2 kb. The 1.15- and 0.8-kb transcripts correspond to the *sarA* P2 and P3 transcripts (6), while the ~0.2-kb transcript is a sRNA corresponding to teg49 (Fig. 2B). Likewise, teg48 was also visualized with a P1 probe (−147 to −1) in Northern blot assays. More specifically, a labeled P1 probe (−147 to −1) yielded four bands, corresponding to *sarA* P2, P3, and P1 transcripts and also teg48 at ~200 nt (Fig. 2C).

Mapping the 196-nt teg49 sRNA within the P3-P1 promoter region. We initially focused on teg49 within the P3-P1 *sarA* promoter region. To map the start site and direction of transcription of teg49, we designed DNA primers from sense and antisense strands in the P3 promoter region (spanning −406 to −147 of the translational start site of *sarA*) for primer extension (Table 2) (Fig. 3A). Primer extension analysis with primer PA-13, representing the antisense strand, produced two clear transcripts, one larger transcript for P3 and a smaller transcript corresponding to the start site of teg49, while primers PS-1, -2, -3, and 4 of the sense strain (Table 2) did not yield any transcript (Fig. 3B). Primers PA-11 and PA-12 of the antisense strand both produced single transcripts, with one larger and one smaller than the corresponding *sarA* P3 transcriptional start site as identified by PA-13 (6). These results indicate that the sRNA teg49 exists and is transcribed in the same direction as the *sarA* P3 transcript.

To determine the exact size of teg49, we mapped the 5′ end of the sRNA by primer extension with a sequencing ladder and the 3′ end by RACE-PCR assay. Using primer PA-13, which produced an additional band on the gel (arrow in Fig. 3C), we mapped the 5′ end of teg49 to nt 667081 of the *S. aureus* N315 genome by primer extension (Fig. 3C). We also mapped the 3′ end of teg49 to nt 666886 in the *S. aureus* N315 genome with the 3′ RACE-PCR assay (Fig. 3D). Thus, the teg49 sRNA, at 196 nt in length, is located within the *sarA* P3-P1 promoter region.

Expression of both P3 and teg49 transcripts is *cshA* and *sigB* dependent. In recent studies, it has been reported that CshA, a DEAD box RNA helicase (26), can alter the stability of the *agr* mRNA in *S. aureus*. To assess if CshA can regulate the expression of sRNA teg49 and its associated *sarA* transcripts, we conducted Northern blot assays of parental strain ALC6094 and its isogenic *cshA* mutant and the complemented mutant with a teg49 probe. As shown in Fig. 4A, neither a *sarA* P3 transcript nor teg49 was readily detectable in the *cshA* mutant during the early, mid-log, and late log phases, in contrast to parental strain ALC6094 and the complemented mutant. In addition, the corresponding P2 transcript level was also lower in the *cshA* mutant than in the parental

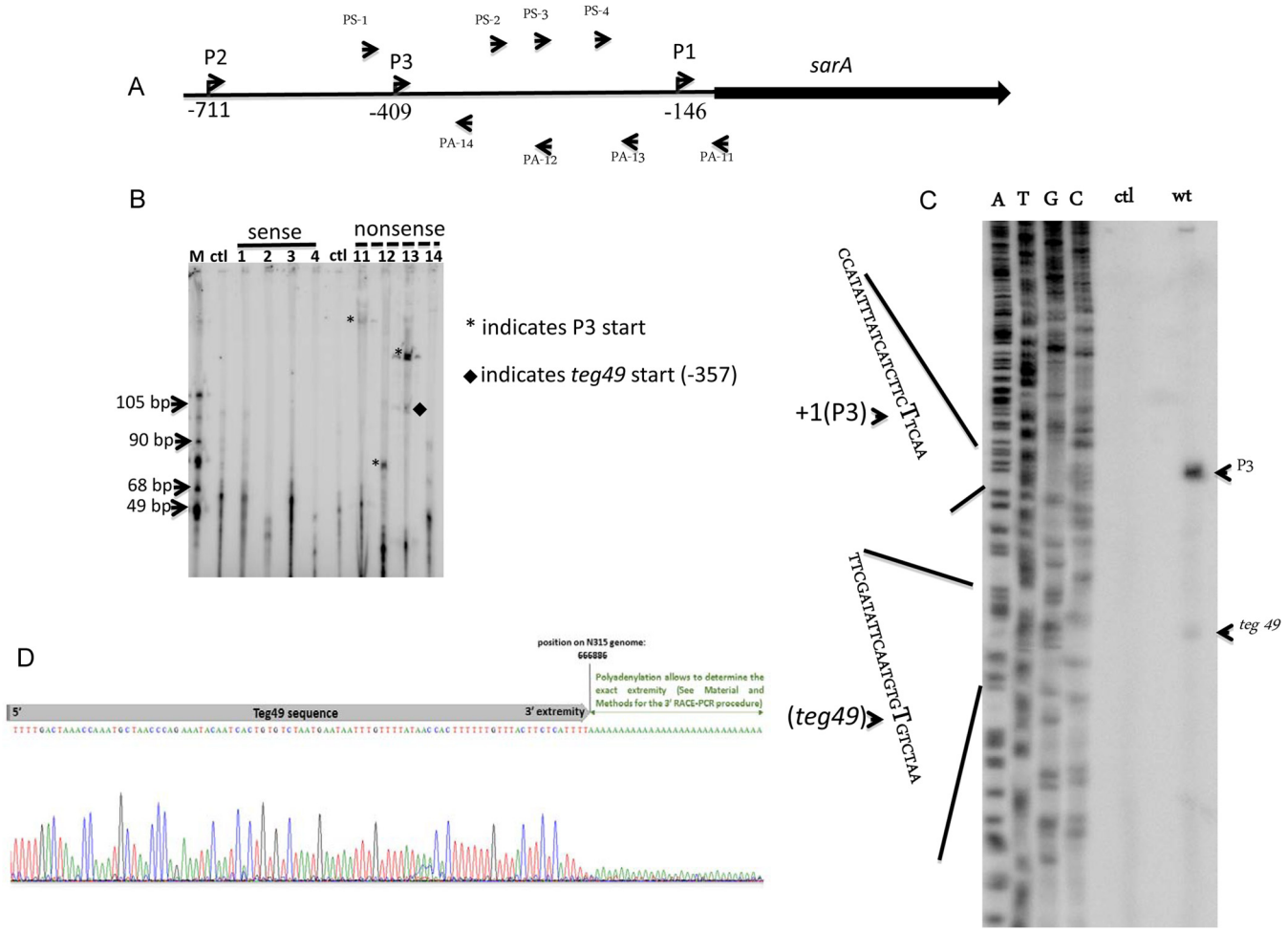

FIG 3 Primer extension studies of two transcripts in the *sarA* P3 promoter region. (A) Map of *sarA* and the 5′ UTR. The directions of transcription of P2, P3, and P1 are indicated by arrowheads. The oligomers used for primer extension are shown above and below the *sarA* genetic map. The directions of the oligomers are indicated by arrowheads. The oligomers were customized from the sense strand (PS-1, PS-2, PS-3, and PS-4) and the antisense strand (PA-11, PA-12, PA-13, and PA-14) (Table 2). (B) Primer extension analysis of teg49 containing putative primer extension products from the sense and nonsense strands, including a control reaction mixture (lane ctl) that contained only total RNA prepared from *S. aureus* ALC6094. The transcriptional start sites of P3 (at −409 from the translational start site of *sarA*) and teg49 (at −357 from the translational start site of *sarA*) are indicated by asterisks and a diamond, respectively. Lane M contained molecular size markers. (C) Primer extension with oligomer PA-13 and total RNA isolated from *S. aureus* ALC6094 grown to exponential phase (OD_{650} of 0.7) in TSB medium as the template. The transcriptional start sites of P3 and teg49 are indicated on the right. A part of the sequence of the P3 promoter deduced from the sequencing reaction is shown on the left; P3 and teg49 starting sites from T's (complementary nucleotides of A's) are indicated by bold letters and arrowheads. wt, wild type. (D) 3′ RACE DNA sequencing reactions indicating the 3′ end of teg49.

and complemented strains. Interestingly, both teg49 and *sarA* P3 transcripts, as detected by Northern blot assays, reemerged in cells grown to the stationary phase (OD_{650} of 1.7) (Fig. 4A), suggesting that factors other than CshA may be important in the generation of sRNA teg49 and the *sarA* P3 transcript during the stationary phase.

To confirm the Northern blot assay results for exponential-phase cells, we conducted primer extension studies of the *cshA* mutant, isogenic parental, and complemented strains at an OD_{650} of 0.7 with primer PA-13. We found weaker primer extension signals for both *sarA* P3 and teg49 in the *cshA* mutant than in the parental and complemented strains (Fig. 4B). More specifically, the *cshA* mutant exhibited notably lower levels of primer extension signals for teg49 (~2.4-fold lower, as determined by densitometry) (Fig. 4C) and the *sarA* P3 transcript (~4.7-fold less) (Fig. 4D) than the isogenic parent.

In previous studies, we and others have shown that *sigB* regulates *sarA* P3 transcript expression in *S. aureus* (27, 28). As SigB is involved in the postexponential stress response and teg49 somehow reemerges in a *cshA* mutant during the stationary phase, we evaluated whether *sigB* is involved in the expression of sRNA teg49. As expected, the *sarA* P3 transcript level was significantly lower in the *sigB* mutant than in parental strain ALC6094 and also the complemented mutant (Fig. 4E). Remarkably, sRNA teg49 was not observed in the *sigB* mutant, even during the stationary phase (OD_{650} of 1.7, Fig. 4E), suggesting that teg49 expression is dependent on *sigB*. Thus, the *sigB* mutant of ALC6094 cannot generate either a *sarA* P3 transcript or sRNA teg49 but the two transcripts reemerged in the complemented *sigB* mutant. In contrast to the *cshA* mutant, the *sigB* mutant did not yield any *sarA* P3 transcript or sRNA teg49 in any of the three growth phases, including the stationary phase (Fig. 4E).

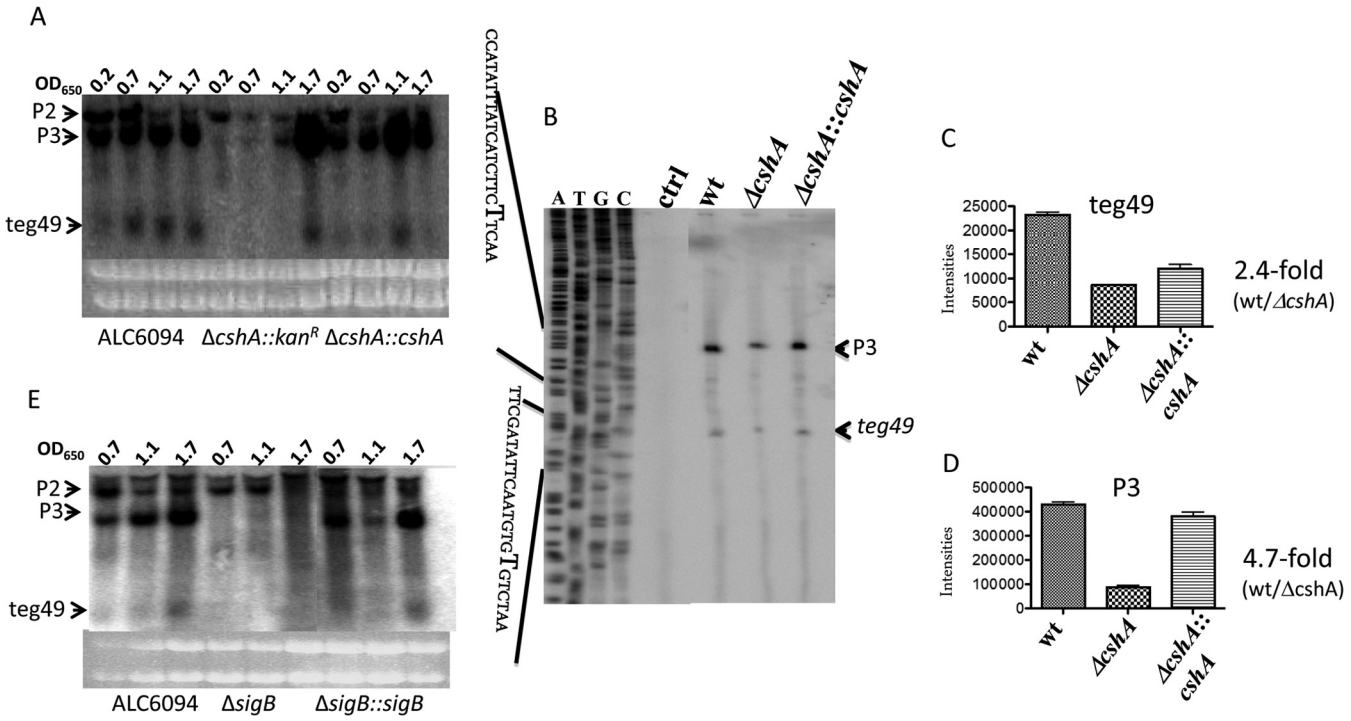

FIG 4 Northern blot analysis of sRNA teg49 in *S. aureus* strains in various phases of growth. (A, E) Northern blot assay detection of sRNA teg49. Total RNAs (10 μg) from Δ*cshA* (A) and Δ*sigB* (E) mutants of strain Newman derivative ALC6094 grown in TSB medium were separated in a 1.5% agarose gel and then transferred to an H^+-bond membrane for probing. sRNA teg49 was detected with a teg49 probe. (B) Primer extension analysis of sRNA teg49 in a *cshA* mutant of ALC6094. Primer extension assays were performed with RNAs purified from the parental (ALC6094), Δ*cshA* mutant, and complemented strains grown to exponential phase (OD_{650} of ~0.7) in TSB medium with oligonucleotide PA-13 (Table 2). ctrl, control; wt, wild type. (C, D) Quantification of *sarA* P3 and teg49 transcripts in the *cshA* mutant, parental, and complemented strains from panel B. Transcripts *sarA* P3 (C) and sRNA teg49 (D) were quantified with ImageJ (NIH). These experiments were repeated at least three times, and one set of typical experiments is shown.

sRNA teg49 has two hairpin structures and sequences conserved among *S. aureus* strains. The RNA structure of teg49 was predicted by the RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) (Fig. 5A). The sRNA teg49 appears to have two stem-loop structures, each with a long stem and a short loop, designated HP1 and HP2. HP1 and HP2 are located at nt 667059 to 667067 and 667018 to 667027 of the *S. aureus* N315 genome (Fig. 5A), respectively. The sequences of teg49 are also highly conserved among methicillin-resistant and -susceptible *S. aureus* strains (Fig. 5B).

HP1 loop mutation of teg49 has a prominent effect on *sarA* P3 expression. Previous experiments have shown that hairpins in noncoding sRNAs are important sites for posttranscriptional gene regulation (12, 13, 14). Given the conservation of teg49 among various *S. aureus* strains, we thus wanted to elucidate the contribution of the loop and stem sequences of HP1 and HP2 on *sarA* expression. In this instance, we elected to work with strain SH1000, which is a version of strain 8325-4 with *rsbU* restored that is more genetically amenable and has been extensively studied. To ensure that teg49 is also present within the *sarA* promoter region of strain SH1000, we conducted a Northern blot assay with a teg49 probe, which showed that teg49, along with P2 and P3 *sarA* transcripts, was easily detectable in this strain (Fig. 6A, first lane). We subsequently created chromosomal transversion mutations of teg49 in SH1000, yielding an HP1 stem mutant (ALC7288), an HP1 loop mutant (ALC7289), an HP2 loop mutant (ALC7290), and an HP2 stem mutant (ALC7291) (Table 1). The expression of teg49 with a teg49 probe appeared to be reduced in two loop mutants, ALC7289 (HP1 loop mutant) and ALC7290 (HP2 loop mutant), but not in two stem mutants (Fig. 6A). Surprisingly, in the HP1 loop mutant, where there is a 7-base transversion mutation in the chromosome, the P3 and P2 transcripts are almost undetectable or are significantly reduced (Fig. 6A). Thus, the HP1 loop in teg49 seems to be critical for *sarA* P3 and teg49 transcription.

To analyze the effect of the HP1 loop mutation of teg49 on *sarA* P2 and P3 transcription more clearly, we ran a lower-percentage agarose gel (0.7% in Fig. 6B versus 1.5% in Fig. 6A) for Northern blot assays with a *sarA* probe (*sarA* coding region) (Fig. 6B). As expected, compared to the parental and complemented strains, the HP1 loop mutant exhibited lower levels of *sarA* P1, P3, and P2 transcript expression. Remarkably, there were two additional bands, one located between the P2 and P3 transcripts (one asterisk in Fig. 6B) and the other located between the P3 and P1 transcripts (two asterisks in Fig. 6B) in the HP1 loop mutant. The origin of these two transcripts is not clear. However, it is plausible that the larger transcript is a truncated form or processed from the P2 transcript while the smaller transcript can be derived from either the P2 or the P3 transcript, indicating that the 7-base HP1 loop sequence is critical to processing of the *sarA* transcripts. More importantly, processing of the *sarA* transcripts is associated with reduced expression of teg49 (Fig. 6A). Notably, complementation of the 7-base mutation in the HP1 loop mutant in the chromo-

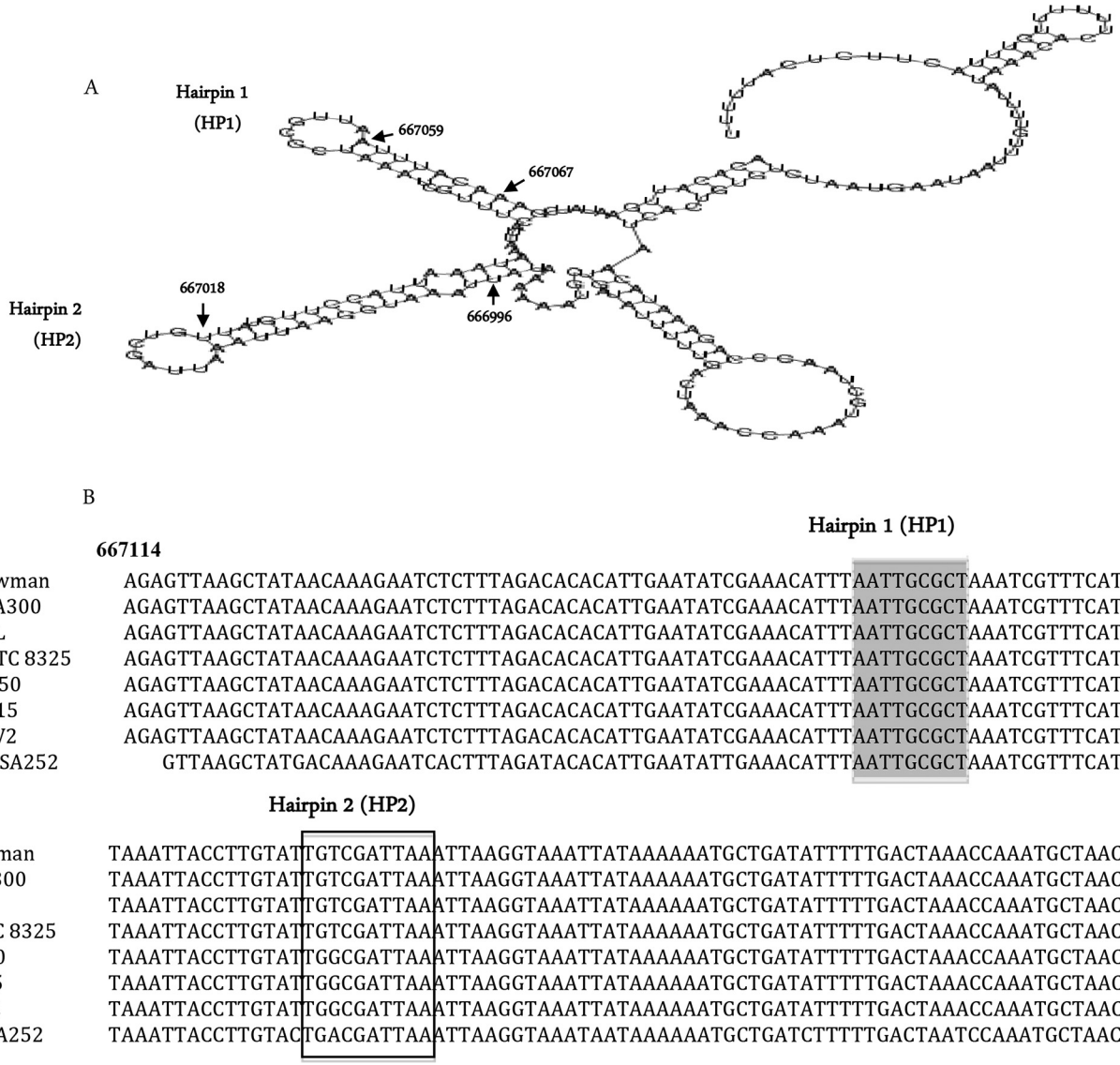

FIG 5 Predicted secondary structure and comparison of the teg49 nucleotide sequences of *S. aureus* strains. (A) Predicted secondary structure of sRNA teg49. The structure of teg49 was predicted by the RNAfold web server (see Results). Two hairpin-loop structures, HP1 and HP2, within teg49 were found to be located at nt 667059 and 667018 of the *S. aureus* N315 genome, respectively. (B) Sequences upstream of the *sarA* coding region bearing putative sRNA teg49 are conserved. A partial alignment of the teg49 sequences of *S. aureus* strains, Newman, USA300, COL, NCTC 8325, Mu50, N315, MW2, and MRSA252 is shown. The HP1 and HP2 hairpin-loop sequences are shaded in gray and boxed, respectively.

some restored the transcription of all three *sarA* transcripts in the mutant (Fig. 6B).

The effect of HP1 loop mutation on *sarA* transcription also includes lower SarA protein expression than in the parental and complemented mutant strains in whole-cell lysates of Western blot assays probed with an anti-SarA monoclonal antibody, but the level of expression was still higher than that of the *sarA* deletion mutant (Fig. 6C). Given that *sarA* is a positive regulator of *agr* expression (29), we conducted Northern blot assays with an *agrA* probe, which showed that RNAII (containing *agrA*) expression was lower in the HP1 loop mutant than in the parental and complemented mutant strains (Fig. 6B). However, the reduction was less than that of a true *sarA* deletion mutant. We next examined the aureolysin gene *aur*, which is negatively regulated by *sarA* (30). As expected, the expression of *aur* was upregulated in the HP1 loop mutant, similar to that in the *sarA* deletion mutant (Fig. 6B). Previous studies have described a critical role for SarA in biofilm formation by *S. aureus* (31, 32). As anticipated for a strain with reduced SarA expression, the HP1 loop mutant also exhibited less biofilm formation than the wild type and the complemented mutant ($P \leq 0.01$ versus the parent by the Student *t* test), but the reduction in biofilm formation was less than that of the *sarA* deletion mutant (Fig. 6D).

Complementation of the HP1 loop mutant in *trans* with pEPSA5 carrying teg49. To complement the HP1 loop mutation in *trans*, we first cloned teg49 into pEPSA5, a shuttle plasmid containing a xylose-inducible promoter in *E. coli*; this was followed by transformation into the HP1 loop mutant. We were able to detect

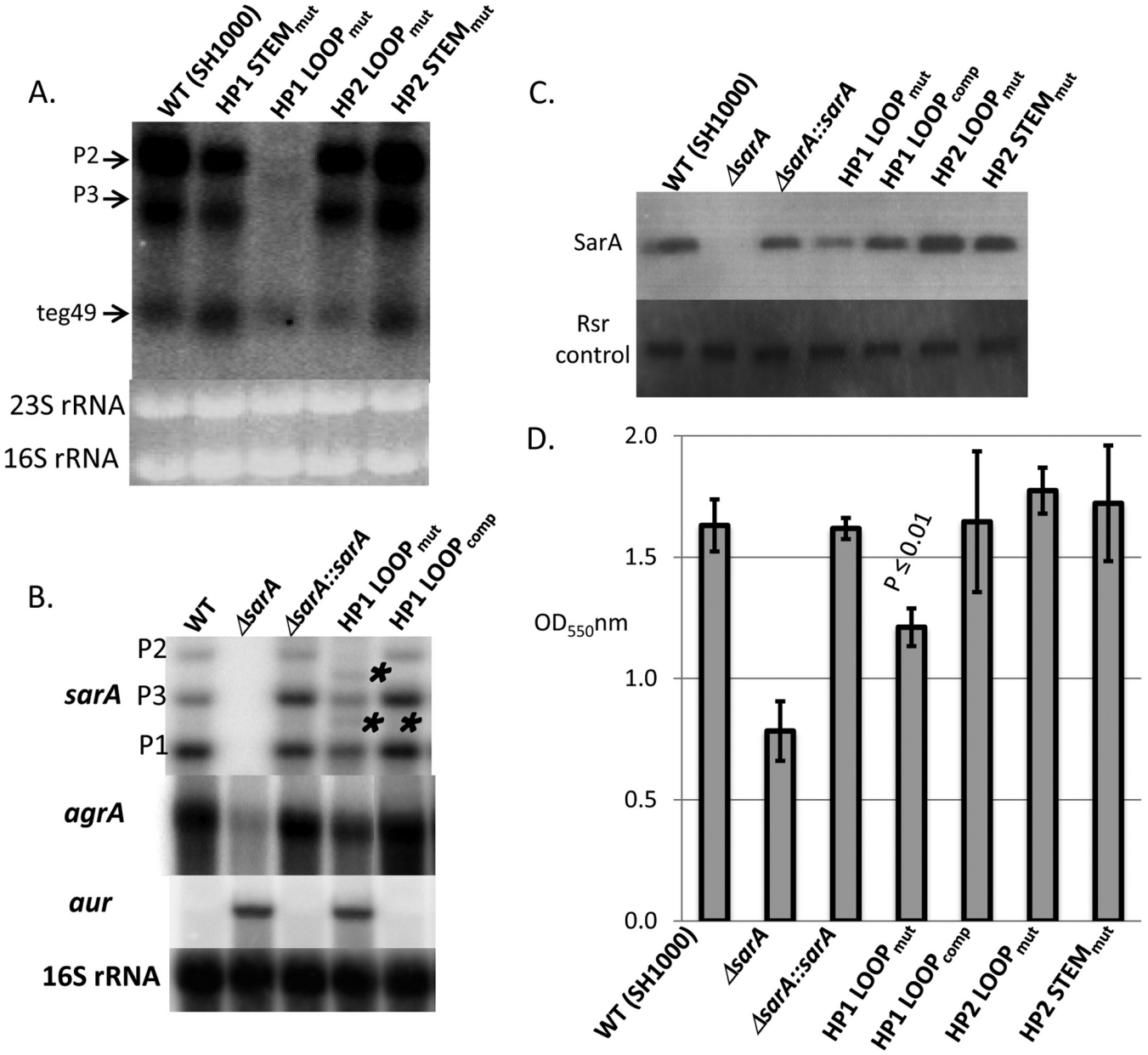

FIG 6 Analysis of HP1 loop mutant with respect to *sarA* P3 and teg49 transcription. (A) Northern blot assay (from a 1.5% agarose gel optimized for sRNA detection) of wild-type (WT) SH1000, HP1 stem and loop mutants (mut), and HP2 stem and loop mutants (from late-log-phase cells at an OD_{650} of 1.1) probed with a labeled teg49 probe. As expected, there are three transcripts from wild-type SH1000, *sarA* P2 and P3 transcripts, and teg49. The *sarA* P1 transcript was not expected to be detected by the teg49 probe. (B) Northern blot assays (from a 0.7% agarose gel to detect transcript larger than 500 bases) of isogenic *sarA* strains along with an HP1 loop mutant and a complemented (comp) HP1 loop mutant. The probes are the *sarA* coding region for *sarA*, *agrA* for RNAII, and *aur* for the aureolysin gene. 16S rRNAs were used as loading controls. We included wild-type SH1000, a *sarA* deletion mutant, and complemented mutants for *sarA* transcripts (P1, P3, and P2 for 0.5, 0.8, and 1.1 kb, respectively) as additional controls. The single and double asterisks represent altered *sarA* transcripts in the HP1 loop mutant. This defect was restored in the complemented HP1 loop mutant (HP1 $LOOP_{comp}$). (C) Western blot assay for SarA in isogenic *sarA* mutant strains with an HP1 loop mutant, a complemented mutant, and HP2 stem and loop mutants. The blot was probed with an anti-SarA monoclonal antibody at 1:1,000, followed by conjugate. Rsr, a cellular protein that was constant during growth, was used as a loading control and was detected by a mouse anti-Rsr antibody. (D) Biofilm formation by assorted *sarA* strains in microtiter wells containing TSB and glucose. Cells were inoculated into wells and grown overnight. Biofilms were stained with crystal violet and solubilized with ethanol, and OD_{550}s were read. *sarA* mutant and complemented mutant strains were used as negative and positive controls, respectively.

teg49 expression in the HP1 loop mutant upon induction with 1% xylose versus the empty-vector control (Fig. 7A). Using cells at an OD_{650} of 1.1 in the presence of 1% xylose, we discovered by Northern blot assays that the *sarA* P3 and P1 transcript levels were significantly enhanced in the HP1 loop mutant upon xylose induction while cells without xylose induction or those with the vector alone did not exhibit similar complementation. Interestingly, the cleaved transcript sizing between the P3 and P1 transcripts in the HP1 loop mutant persisted in the HP1 loop mutant carrying pEPSA5::teg49, consistent with the combination of de-

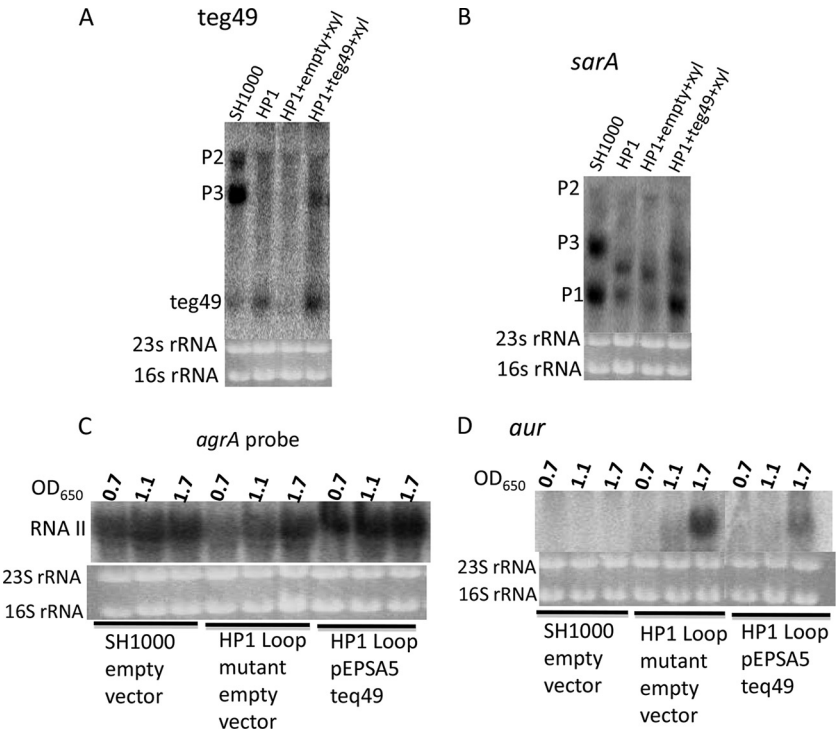

FIG 7 Northern blot analysis of *S. aureus* HP1 loop mutant containing pEPSA-teg49 in the presence of 1% xylose. An *S. aureus* HP1 loop mutant harboring pEPSA5-teg49 or control empty pEPSA5 was grown in TSB containing 1% xylose at 37°C, and cells were harvested at late exponential phase (OD_{650} of 1.1) (A, B) and at three different growth stages (OD_{650}s of 0.7, 1.1, and 1.7) (C,D). Purified total RNAs (10 μg/lane) were separated in a 1.5% agarose gel, transferred to H^+-bond membranes, and then hybridized with radiolabeled DNA probes for teg49 (A), *sarA* (B), *agrA* (C), and *aur* (D).

fective and normal HP1 loops of teg49 in this strain. Importantly, the RNAII transcript, as detected by the *agrA* probe, was intensified in the HP1 loop mutant carrying pEPSA5::teg49 (Fig. 7C). Likewise, the aureolysin gene *aur* was more repressed in the HP1 loop mutant with pEPSA5::teg49 than in cells with only the vector control (Fig. 7D). Taken together, these data showed that teg49 is likely a *trans*-acting sRNA that is capable of complementing the defect in *sarA* transcription in the HP1 loop mutant.

DISCUSSION

The *sarA* locus encompasses the 372-bp *sarA* coding region preceded by a long ~800-bp 5′ UTR that adopts a three-promoter system responsible for modulating the abundance of the SarA protein. SarA is a pleiotropic regulator of virulence, oxidative stress, and biofilm formation in *S. aureus* (1, 2, 23, 31, 32, 33). In this study, we also detected two sRNAs in the 5′ UTR of the *sarA* locus and found that sRNA teg49 likely contributes to the virulence of *S. aureus* by modulating SarA expression. This report thus adds to the growing list of sRNAs involved in the control of bacterial virulence in *S. aureus*, including RNAIII, SprD, RsaE, SprA1, SSR42, and ArtR (12, 13, 14, 15, 34, 35). Unlike the orientation of many of the antisense sRNAs, teg49 is in the same direction as the *sarA* P1, P3, and P2 transcripts, as determined by RNA-Seq and primer extension studies (Fig. 1B). In the absence of any indication of a riboswitch, teg49 is likely a *trans*-acting sRNA. We were able to detect teg49 by Northern blot assays with a radiolabeled P3 (data not shown) and/or teg49 DNA probe but not with the *sarA* P1 and P2 and *sarA* ORF probes (data not shown), consistent with the location of teg49 between the *sarA* P3 and P1 promoter regions, as verified by mapping studies.

The expression of sRNA teg49 appears to be *sigB* dependent. However, we did not find a *sigB*-dependent promoter (GGGTAT at the −10 position) immediately upstream of the transcription start site. Notably, the absence of teg49 from a *sigB* mutant coincides with a lack of activation of the SigB-dependent *sarA* P3 promoter (27, 28) in a *sigB* mutant, while *sarA* P3 transcript restoration was accompanied by the reemergence of teg49 in a complemented *sigB* mutant (Fig. 4E). In addition, as expected with a *sigB*-dependent transcript (i.e., *sarA* P3 transcript) (36, 37), teg49 was maximally expressed during the stationary phase (Fig. 4E). Finally, erratic processing of the P2 and P3 *sarA* transcripts in the HP1 loop mutant also reduced teg49 expression (Fig. 6A). Taking our findings together, we conclude that teg49 does not have a promoter and is likely derived from the *sigB*-dependent *sarA* P3 transcript.

We have shown that the expression of teg49 is *cshA* dependent. CshA is an ATP-dependent DEAD box RNA helicase that unwinds duplex RNA. CshA has been known to be involved in ribosome biogenesis, ribosome assembly, and mRNA decay in a degradosome involving RNase J and Y and polynucleotide phosphorylase (PNPase) (26). However, the protection (or stability) of sRNAs such as teg49 by CshA has not been previously described. In addition, RNase E is absent from *S. aureus*. In a separate study, we recently analyzed the transcriptome of a *cshA* mutant of ALC6094, showing that CshA is indeed required for the preservation and/or stability of at least 15 sRNAs in the *S. aureus* Newman RNome (unpublished data). In contrast to the regulation of the *sigB* mutant, regulation of teg49 by *cshA* is active only from early to late log phase since teg49 expression reemerged in the *cshA* mutant during stationary phase (OD_{650} of 1.7) (Fig. 4A).

In silico analysis indicated that teg49 forms two hairpin structures with small loops, which we called HP1 and HP2 (Fig. 5A). These secondary structures are often the site of binding to other mRNAs or proteins. To confirm the importance of these secondary structures, we performed transversion mutations of the stem and loop sequences of HP1 and HP2. Mutational analysis of HP1 of teg49 indicated that the HP1 loop has a modulatory function important for controlling virulence gene expression by regulating SarA protein expression (Fig. 6C). In comparison to the parental strain, the HP1 loop mutant with a 7-bp replacement (ATTGCG C→CGGTATA) in the chromosome not only exhibited tapered expression of *sarA* P2, P3, and teg49 transcripts (Fig. 6A and B) but also led to the formation of two truncated transcripts. The larger truncated transcript is likely derived from the *sarA* P2 transcript, while the origin of the smaller truncated transcript is not clear and may be the P2 or P3 transcript (Fig. 6B, asterisks). A consequence of the modulation of *sarA* P2 and P3 transcripts as a result of the HP1 loop mutation is reduced synthesis of the SarA protein, resulting in the dysregulation of downstream genes, including increased *aur* but decreased RNAII expression (Fig. 6B). Importantly, this has also led to reduced biofilm formation *in vitro* in the HP1 loop mutant, but the level of reduction is less than that of the *sarA* deletion mutant.

We have noticed that expression of teg49 was lower in the HP1 loop mutant than in the parent (Fig. 6A). To verify that teg49 can act in *trans* to complement the HP1 loop mutant, we confirmed that *sarA* P3 and P1 transcription can be restored close to the parental level in this mutant by expressing teg49 from a xylose-inducible promoter in pEPSA5. Given that the orientation of teg49 is identical to that of the *sarA* transcripts, we ruled out teg49 as a *cis*-acting sRNA. In the absence of a riboswitch structure and the ability to complement in *trans*, we concluded that teg49 is likely a *trans*-acting sRNA. On the basis of the studies reported here, we propose that 5′-terminal stem-loop HP1 of teg49 is critical in contributing to the stability of *sarA* P3 and possibly other *sarA* transcripts. This hypothesis also implies that the HP1 loop sequence in teg49 may be important in preventing erratic processing by host RNases, presumably via an mRNA binding protein that confers protection from defective processing. One plausible candidate for this protection may be CshA, which is likely involved in the generation of teg49 (Fig. 4A) and possesses presumably RNA binding activity (38, 39, 40). Further investigations are needed to verify this possibility.

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