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The *Staphylococcus aureus* Protein-Coding Gene *gdpS* Modulates *sarS* Expression via mRNA-mRNA Interaction

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***Staphylococcus aureus* is an important Gram-positive pathogen responsible for numerous diseases ranging from localized skin infections to life-threatening systemic infections. The virulence of *S. aureus* is essentially determined by a wide spectrum of factors, including cell wall-associated proteins and secreted toxins that are precisely controlled in response to environmental changes. GGDEF domain protein from *Staphylococcus* (GdpS) is the only conserved staphylococcal GGDEF domain protein that is involved not in c-di-GMP synthesis but in the virulence regulation of *S. aureus* NCTC8325. Our previous study showed that the inactivation of *gdpS* generates an extensive change of virulence factors together with, in particular, a major Spa (protein A) surface protein. As reported, *sarS* is a direct positive regulator of *spa*. The decreased transcript levels of *sarS* in the *gdpS* mutant compared with the parental NCTC8325 strain suggest that *gdpS* affects *spa* through interaction with *sarS*. In this study, site mutation and complementary experiments showed that the translation product of *gdpS* was not involved in the regulation of transcript levels of *sarS*. We found that *gdpS* functioned through direct RNA-RNA base pairing with the 5′ untranslated region (5′UTR) of *sarS* mRNA and that a putative 18-nucleotide region played a significant role in the regulatory process. Furthermore, the mRNA half-life analysis of *sarS* in the *gdpS* mutant showed that *gdpS* positively regulates the mRNA levels of *sarS* by contributing to the stabilization of *sarS* mRNA, suggesting that *gdpS* mRNA may regulate *spa* expression in an RNA-dependent pathway.**

Staphylococcus aureus is a significant opportunistic pathogen that causes a broad spectrum of infections, ranging from localized skin infections to life-threatening systemic infections (1, 2). A wide range of virulence factors of *S. aureus* are precisely controlled in response to various environmental and physiological demands (3, 4), including secreted toxins such as hemolysins (alpha, beta, gamma, and delta), enterotoxins, coagulase, nucleases, lipase, and proteases and cell wall-associated proteins such as fibronectin-binding protein, fibrinogen-binding protein, clumping factor A, clumping factor B, and Spa (5, 6).

Spa is a surface matrix binding protein which is expressed during the exponential phase and is transcriptionally downregulated during the postexponential phase of growth (7, 8). Previous studies have shown that Spa binds to the Fc regions of immunoglobulin G (IgG) in several mammalian species and is thought to be important in phagocytosis avoidance (9, 10). In *S. aureus*, a variety of regulatory factors have been shown to regulate *spa* expression, including the accessory gene regulator (Agr) quorum-sensing global regulatory system and DNA-binding proteins such as SarA, SarS, SarT, and Rot (3, 11).

Among these regulatory factors, SarA represses the expression of *spa*, which comprises a primary global regulatory system with the *agr* loci (3). As one of the SarA homologs, SarS (initially designated SarH1) is located on the chromosome between the staphylococcal iron-regulated transporter (*sirABC*) and *spa*, upregulates *spa* expression by directly binding to the *spa* promoter, and appears to play a key role in this regulatory network (12, 13).

3′,5′-Cyclic diguanylic acid GMP (c-di-GMP) was first identified as an allosteric activator of cellulose synthase in *Gluconacetobacter xylinus* (14). Further studies have shown that c-di-GMP is a ubiquitous bacterial second messenger and is involved in biofilm formation and the modulation of virulence in several pathogens. C-di-GMP is synthesized from two GTP molecules by a class of enzymes called diguanylate cyclases (DGCs), which contain GGDEF domains, and is hydrolyzed, respectively, into pGpG or two GMPs by members of another protein family named phosphodiesterases (PDEs) containing EAL or HD-GYP domains (15–17). Typically, the numbers of proteins that contain GGDEF, EAL, and/or HD-GYP domains are different in different bacteria (18). In *S. aureus* NCTC8325, only one conserved GGDEF domain-containing protein, GdpS, has been identified, while no EAL domain-containing proteins have been reported (4, 19, 20).

Recent studies have shown that GdpS in both *S. epidermidis* and *S. aureus* cannot synthesize c-di-GMP, suggesting that staphylococci may have only remnants of a c-di-GMP signaling pathway (19, 21). Our previous research has found that *gdpS* affects transcriptional profiling in *S. aureus* NCTC8325 and also regulates a wide range of virulence factors, in particular, the transcription of *spa* through SarS. The influences of GdpS on *spa* and *sarS* depend on its N-terminal domain rather than its C-terminal do-

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TABLE 1 Bacterial strains and plasmids used in this study[a]

Strain or plasmid	Description	Source
Strains		
S. aureus NCTC8325	Wild type	NARSA
S. aureus RN4220	8325-4, r$^-$	NARSA
S. aureus gdpS mutant	8325 *gdpS*::*ermB*	This study
S. aureus C-*gdpS*	8325 *gdpS*::*ermB* (pLIgdpS)	This study
S. aureus C/ATG-M	8325 *gdpS*::*ermB* (pLIgdpS; "ATG" mutant)	This study
S. aureus C/ATG-DEL	8325 *gdpS*::*ermB* (pLIgdpS; "ATG" deletion)	This study
S. aureus C/mutant1	8325 *gdpS*::*ermB* (pLIgdpS; "UAAUUUUGUCACUGUAU" of mutant of the putative 18-nt region)	This study
S. aureus C/mutant2	8325 *gdpS*::*ermB* (pLIgdpS; "UAAUUUUG" mutant of the putative 18-nt region)	This study
S. aureus C/mutant3	8325 *gdpS*::*ermB* (pLIgdpS; "UCAC" mutant of the putative 18-nt region)	This study
S. aureus C/mutant4	8325 *gdpS*::*ermB* (pLIgdpS; "GUAU" mutant of the putative 18-nt region)	This study
E. coli DH5α	Host strain for cloning	Host strain for cloning
Plasmids		
pEASY-TB	Cloning vector; Kan^r Ap^r	TransGen
pLI50	Shuttle cloning vector; Ap^r Cm^r	Addgene
pLIgdpS	pLI50 with *gdpS* and its promoter; Ap^r Cm^r	This study
pLIlyt	pLI50 with N-terminal 5TMR-LYT domain and its promoter; Ap^r Cm^r	This study
pLIgdpSATG-M	pLIgdpS with a mutation of the initiation codon (ATG to TAG)	This study
pLIgdpSATG-D	pLIgdpS with a deletion of the initiation codon	This study
pLIgdpSm1	pLIgdpS with a mutation of the putative 18-nt region (UAAUUUUGUCACUGUAU to AUUAAAACAGUGACAUA)	This study
pLIgdpSm2	pLIgdpS with a mutation of the putative 18-nt region (UAAUUUUG to AUUAAAAC)	This study
pLIgdpSm3	pLIgdpS with a mutation of the putative 18-nt region (UCAC to AGUG)	This study
pLIgdpSm4	pLIgdpS with a mutation of the putative 18-nt region (GUAU to CAUA)	This study
pEAgdpS	pEASY TB with *gdpS* fragment for *in vitro* transcription	This study
pEAsarS	pEASY TB with *sarS* fragment for *in vitro* transcription	This study
pEAspa	pEASY TB with *spa* fragment for *in vitro* transcription	This study
pEArgdpS	pEASY TB with reverse *gdpS* fragment for *in vitro* transcription	This study

[a] Ap, ampicillin; Kan, kanamycin; Cm, chloramphenicol; NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*; r$^-$, restriction deficient.

main, suggesting that GdpS functions in *S. aureus* via an unknown mechanism, independently of c-di-GMP signaling (20).

In this study, further experiments were carried out to interpret the detailed mechanism whereby *gdpS* regulates *sarS* and *spa*. Our data indicate that *gdpS* regulates the mRNA levels of *sarS* through mRNA-mRNA interactions. By performing site mutation and complementary experiments, we showed that *gdpS* regulates the mRNA levels of *sarS* independently of the translation product of *gdpS*. The gel-shift analysis demonstrated that *gdpS* mRNA directly binds to *sarS* mRNA *in vitro*. In addition, mRNA half-life analysis indicated that *gdpS* positively regulates *sarS* mRNA levels by contributing to the stabilization of *sarS* mRNA.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. Constructed plasmids were transformed into RN4220 as the initial recipient and then NCTC8325 by electroporation. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium with appropriate antibiotics (ampicillin, 100 μg/ml; kanamycin, 50 μg/ml). *S. aureus* and its derivative strains were grown in tryptic soy broth (TSB) medium with erythromycin (2.5 μg/ml) and chloramphenicol (15 μg/ml) if necessary. The media were solidified with 1.5% (wt/vol) agar when required.

Complementation of the *gdpS* mutant. The different fragments of the *gdpS* gene and its native promoter from *S. aureus* NCTC8325 were amplified and ligated into shuttle plasmid pLI50, respectively. Plasmids with a mutated translation initiation codon and different mutated nucleotides ranging from 18 nucleotides (nt) to 4 nt (see Fig. 4A) were constructed by site-directed mutagenesis (22). Using corresponding complementing plasmids as the templates for PCR, DNA fragments containing full-length plasmids were amplified. The PCR products were digested with DpnI to remove the template plasmids and then phosphorylated, self-ligated, and subsequently transformed into *E. coli* DH5α. Complementing plasmids were transformed into *S. aureus* RN4220 for modification and, subsequently, transformed into relevant mutant strains. The primers used in this study are listed in Table 2.

RNA isolation, cDNA generation, and real-time reverse transcription-PCR (RT-PCR). For total RNA isolation, overnight cultures of *S. aureus* were inoculated at 1:100 in LB medium and grown to various growth phases (3 to 8 h). RNA extraction was performed as described by Wolz et al. (23). The cells were pelleted and lysed in 1 ml RNAiso (TaKaRa) with 0.1-mm-diameter zirconia-silica beads in a FastPrep-24 automated system (MP Biomedicals), and residual DNA was degraded with RNase-free DNase I (TaKaRa).

Reverse transcription was carried out with a PrimeScript 1st Strand cDNA synthesis kit (TaKaRa) using random primers. Real-time PCR was

TABLE 2 Oligonucleotide primers used in this study

Primer name	Oligonucleotide sequence[a]
RT-sarS-f	TTCAATATCTGAAGAACAACGAG
RT-sarS-r	TGAGGGTATTTATGGTGGATT
RT-spa-f	AAGATGGTAACGGAGTACATGTCG
RT-spa-r	TAATAACGCTGCACCTAAGGCTAA
RT-16S-f	CGTGGAGGGTCATTGGA
RT-16S-r	CGTTTACGGCGTGGACTA
muta-gdpATG-f	GTCTGAATCGACTCCTTAATGAC
muta-gdpATG-r	TAGTTCGAACATTTATATACAATA
muta-gdpS18nt-f1	TAATTTTGTCACTGTATAGCCTTAGAAAAAACCATAC
muta-gdpS18nt-f2	TAATTTTGAGTGACATAAGCCTTAGAAA
muta-gdpS18nt-f3	TCACACATAAGCCTTAGAAAAAAC
muta-gdpS18nt-f4	GTATAGCCTTAGAAAAAACCATAC
muta-gdpS18nt-r1	GACAATCGTATCATTATTAT
muta-gdpS18nt-r3	GTTTTAATGACAATCGTATC
muta-gdpS18nt-r4	TCACTGTTTTAATGACAATC
vitro-gdpS-f	GTCAATTAAAGGAGTCGA
vitro-gdpS-r	GCGGGATCCGATTAACAGTTTTGTCGT
vitro-sarS-f	TATATTAAATAAAGTGCAT
vitro-sarS-r	GCGGGATCCAAATAGAAAACACAAGTGCA
vitro-spa-f	TTTACAAATACATACAGGGG
vitro-spa-r	GCGGGATCCTTATAGTTCGCGACGACGTCC
vitro-gdpS-reverse-f	GGATCCATCTATTTATTCCATCG
vitro-gdpS-reverse-r	ACAATTGCTACAATTACTGTGG
Northern-probe-biotin-N terminus	GGATAGGGTAGACAGATAATAATAATGATACGATTGTCATTAAAACAGTGACATAAGCC
Northern-probe-biotin-C terminus	CACCATATCGTCTGCATCTTTAAACACTTTACGCTGTGATTTTGGATCGTCATCTGTT

[a] Underlining indicates restriction endonuclease recognition sites.

performed with SYBR Premix *Ex Taq* (TaKaRa) using a StepOne real-time PCR system (Applied Biosystems). The quantity of cDNA measured was normalized to the 16S rRNA cDNA abundance. The primers used in this study are listed in Table 2.

Western blot analysis of GdpS. Western blot analysis was performed as previously described (24, 25). Bacterial cells at the early log phase (optical density at 600 nm [OD_{600}] = 1.0) were collected and lysed for 30 min at 37°C by the use of lysostaphin and were then heated for 10 min at 95°C as the sample for electrophoresis. The samples were separated by 12% SDS-PAGE and then electrotransferred onto a polyvinylidene difluoride membrane (GE). The protein was detected by a rabbit anti-GdpS antibody followed by horseradish peroxidase-conjugated sheep anti-rabbit antibodies (Pierce). Polyclonal antibody against GdpS was acquired from Abgent Biotechnology (Suzhou, China), using SVYPIPYREDYLIHLTF peptide as the antigen.

Construction of the *lacZ* reporter vector. The fragment of the *sarS* native promoter and 5′ untranslated region (5′UTR) spanning nucleotides −329 to +18 was amplified from *S. aureus* NCTC8325 and ligated into the pOS1-lacZ plasmid. Plasmids with mutated nucleotides were constructed through the site-directed mutagenesis method as described above. Constructed plasmids were transformed into *E. coli* DH5α for amplification and *S. aureus* RN4220 for modification and, subsequently, were transformed into *S. aureus* NCTC8325. The primers used in this study are listed in Table S2 in the supplemental material.

β-Galactosidase activity assays. For β-galactosidase activity assays, the wild type and the *gdpS* mutant strains with reporter plasmids were collected at the early log phase (OD_{600} = 1.0) and lysed for 20 min at 37°C by the use of 100 μl ABT LSA buffer (60 mM K_2HPO_4, 40 mM KH_2PO_4, 100 mM NaCl, 0.01% Triton X-100, 50 μg/ml lysostaphin). Then, 100 μl ABT buffer and 100 μl ONPG (*o*-nitrophenyl-β-D-galactopyranoside; 4 mg/ml) were added to initiate the reaction. The samples were incubated at 37°C until a yellow color became apparent, and 1 ml Na_2CO_3 was added to stop the reaction. Sample absorbance was read at 420 nm. Units were calculated using the following formula: units = $(1{,}000 \times OD_{420})/(T \times V \times OD_{600})$. *T* (measured in minutes) was the time of incubation, and *V* (in milliliters) was the volume of culture used in the assay.

RACE analysis. The 5′ and 3′ ends of *gdpS* were determined by rapid amplification of the cDNA ends (RACE) using 3′-full RACE core set ver.2.0 and a 5′-full RACE kit (TaKaRa) as previously described (25, 26).

Northern blot analysis. For Northern blot analysis, total bacterial RNA (30 or 150 μg) was processed using 5% polyacrylamide–7 M urea gel electrophoresis and 1× Tris-borate-EDTA (TBE), and then the reaction mixture was transferred to a charged nylon membrane (Millipore) in 0.5× TBE and immobilized by UV cross-linking.

Biotin-labeled oligonucleotide probes for *gdpS* were chemically synthesized (Invitrogen). RNA-DNA hybridization detection using a North2South chemiluminescent hybridization and detection kit (Pierce) was performed to detect *gdpS* transcripts.

***In vitro* transcription and gel-shift assays.** For the gel-shift assays, the *sarS* and *spa* promoters were identified as previously described (11). *In vitro* transcription was carried out with RiboMAX large-scale RNA production systems (Promega). *gdpS* mRNA was amplified and biotin labeled with a Roche biotin RNA-labeling mix (Roche). Labeled probes were incubated with increasing amounts of *sarS* mRNA or *spa* mRNA at 85°C for 2 min and 37°C for 30 min. Then, the mixture was processed using a 4% native polyacrylamide gel and transferred to a charged nylon membrane. Biotin-labeled probes were detected using a North2South chemiluminescent hybridization and detection kit (Pierce).

mRNA half-life assays. Overnight cultures of *S. aureus* were inoculated at 1:100 into LB medium and grown for 3 h. Then, cultures were treated with rifampin (200 μg/ml), which has been reported to rapidly and completely block *S. aureus* mRNA synthesis (27), for 0, 3, 5, or 10 min. Cells were removed after rifampin treatment and processed for RNA isolation, and then the *sarS* mRNA levels were measured by real-time RT-PCR assays.

RESULTS

***gdpS* modulates the mRNA levels of *sarS* independently of its protein form.** According to the results of sequence analysis, *gdpS* encodes a protein comprising two domains: the C-terminal conserved GGDEF domain and the N-terminal 5TMR-LYT domain. Our previous work found that GdpS regulates the expression of Spa through SarS depending on its N-terminal domain rather than its C-terminal GGDEF domain with conserved GGDEF (20). The predicted structure of the N-terminal domain contains several transmembrane regions, and five of these transmembrane regions form a 5TMR-LYT domain, which has been proposed to be the sensor of the LytS-YhcK-type histidine protein kinase. There has been no evidence showing how a transmembrane sensor domain directly regulates gene transcription without participation of the activator domain. Therefore, we speculated that *gdpS* regulates downstream genes in its RNA form rather than the protein form.

In order to determine whether the protein form of *gdpS* plays a role in the regulation of *sarS*, the transcript levels of *sarS* in the wild-type strain, the *gdpS* mutant, and complementary strains were compared. Three kinds of complementary plasmids encoding the sequences of the whole GdpS, the GdpS with mutated ATG (ATG to UAG), and the GdpS with the deletion of ATG were constructed and transformed into the *gdpS* mutant (Fig. 1A). The expression levels of GdpS in the wild-type strain, the *gdpS* mutant, and the three complementary strains were detected by performing Western blot analysis. As expected, only the plasmids containing the whole GdpS sequence restored the GdpS expression level, whereas the expression of GdpS proteins was not detected in the other two complementary strains (Fig. 1B). Subsequently, the transcript levels of *sarS* in the three complementary strains were compared with those in the wild-type strain by performing real-time RT-PCR analysis. Interestingly, the transcript levels of *sarS* in the three complementary strains were all restored to the level seen in the wild-type strain (Fig. 1C), indicating that the regulation of *gdpS* on *sarS* is independent of the translation product of *gdpS*. Therefore, it can be concluded that the protein form of *gdpS* exhibits no function in *sarS* regulation.

Determination of the RNA transcript forms of *gdpS*. Northern blot analysis was performed to determine the RNA transcript forms of *gdpS*. To obtain all the RNA transcripts, we created two 58-bp biotin-labeled probes, which were complementary to the 3′ or 5′ end of *gdpS*, respectively (Fig. 2A). The probes were hybridized to the whole RNA of *S. aureus* NCTC8325. As shown in Fig. 2B, only whole *gdpS* mRNA was detected, ruling out the existence of other small RNAs transcribed by *gdpS*. Meanwhile, the antisense strand of *gdpS* was tested, and no transcript was observed (see Fig. S1 in the supplemental material). The results led us to speculate that *gdpS* mRNA regulated the downstream gene. In addition, we performed primer extension and RACE analysis to determine the precise sequences of *gdpS* mRNA. Sequence analysis indicated that the *gdpS* mRNA contains 1,097 nt.

***gdpS* mRNA binds to *sarS* mRNA *in vitro*.** By sequence alignments, we found an 18-nt region that represents the possible binding site of *gdpS* mRNA with low energy in the 5′UTR of *sarS* mRNA (Fig. 3A). We further performed gel-shift assays to determine whether *gdpS* mRNA binds to *sarS* mRNA, with the reverse transcript of *gdpS* as the positive control. As shown in Fig. 3B, like the positive control, *sarS* mRNA formed complexes with biotin-labeled *gdpS* mRNA, indicating that *gdpS* mRNA can bind to *sarS* mRNA. Furthermore, the 18-nt mutational *sarS* mRNA was transcribed *in vitro* and gel-shift assays were performed to determine whether *gdpS* mRNA was able to bind to the mutational *sarS* mRNA. The results showed that the mutational *sarS* mRNA was no longer able to bind to the biotin-labeled *gdpS* mRNA (Fig. 3C), suggesting that the 18-nt sequence is the binding site between *gdpS* mRNA and *sarS* mRNA.

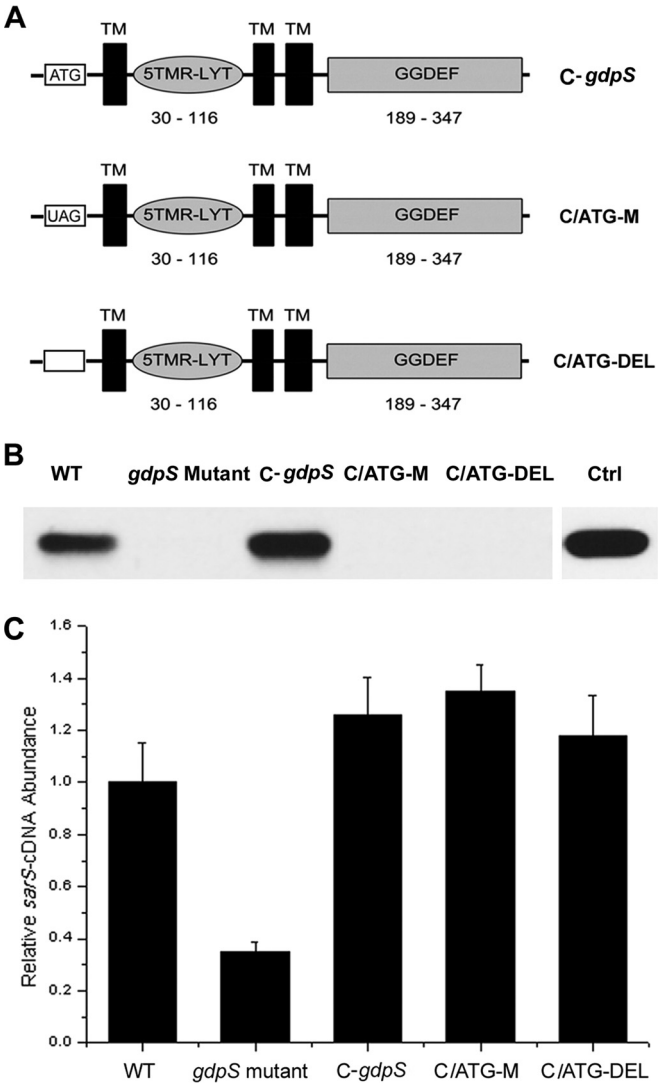

FIG 1 Regulation of *sarS* is independent of the protein form of *gdpS*. (A) The maps of the domain structure of GdpS in the complementary strains C-*gdpS* (the *gdpS* mutant with a plasmid containing whole *gdpS*), C/ATG-M (the *gdpS* mutant with a plasmid containing the *gdpS* gene with mutated ATG), and C/ATG-DEL (the *gdpS* mutant with a plasmid containing the *gdpS* gene with the deletion of ATG). TM, transmembrane region. (B) Western blot analysis of GdpS in the wild type (WT), the *gdpS* mutant, and mutants C-*gdpS*, C/ATG-M, and C/ATG-DEL. Ctrl, control. (C) Real-time RT-PCR analysis of the *sarS* mRNA levels in the wild type, the *gdpS* mutant, and mutants C-*gdpS*, C/ATG-M, and C/ATG-DEL.

The effect of *gdpS* on *sarS* relies on the putative 18-nucleotide region. Since gel-shift assays confirmed the interaction between *gdpS* and *sarS* mRNAs *in vitro*, we were interested in the role played by the putative 18-nucleotide region *in vivo*. Four

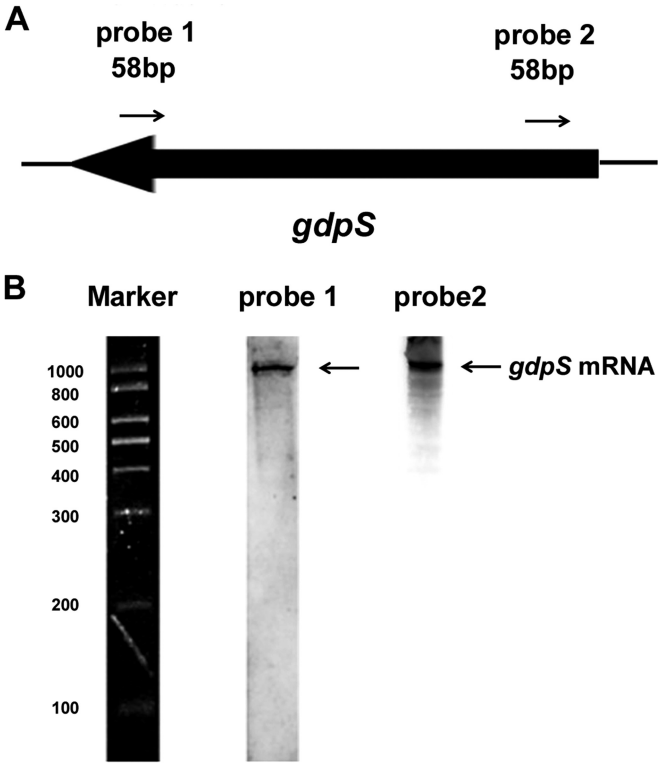

FIG 2 Northern blot assay of *gdpS* in *S. aureus* NCTC8325. (A) Two biotin-labeled probes complementary to the 3′ or 5′ end of *gdpS* were selected. (B) The probes were hybridized to the whole RNA of *S. aureus* NCTC8325 by Northern blotting, and the results show that only the full-length *gdpS* mRNA was detected.

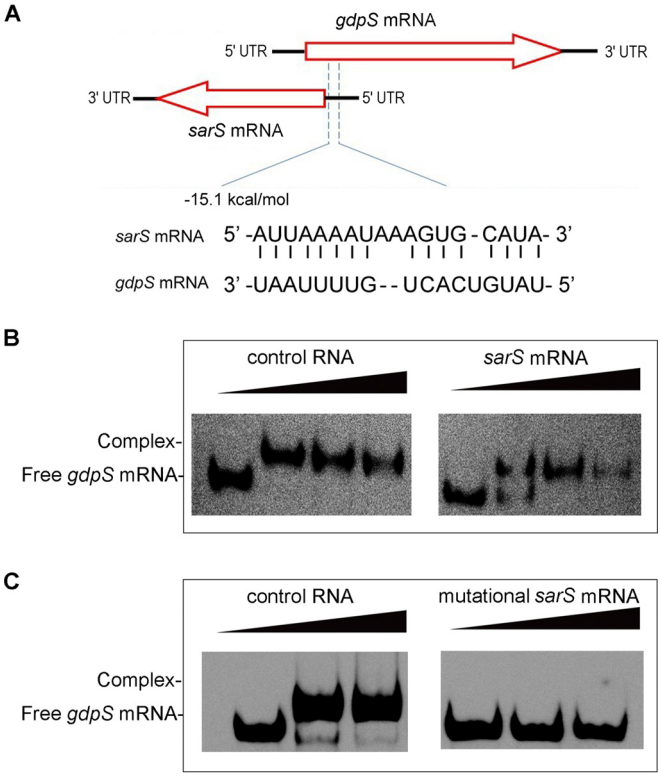

FIG 3 The *gdpS* mRNA binds to *sarS* mRNA *in vitro*. (A) Predicted base pairings between *gdpS* mRNA and *sarS* mRNA. The minimum free energy value is given. (B) The ability of *gdpS* mRNA to bind to *sarS* mRNA was determined by RNA-RNA gel-shift assays. Biotin-labeled *gdpS* mRNA probes were used in all reactions. The antisense RNA was used as a positive control. Increasing amounts of *sarS* mRNA were incubated with the biotin-labeled *gdpS* mRNA probes. (C) The ability of *gdpS* mRNA to bind to the mutational *sarS* mRNA was determined by RNA-RNA gel-shift assays. The predicted binding sequences were mutated in the mutational *sarS* mRNA. Biotin-labeled *gdpS* mRNA probes were used in all reactions, and the *gdpS* antisense RNA was used as a positive control. Increasing amounts of mutational *sarS* mRNA were incubated with biotin-labeled *gdpS* mRNA probes.

kinds of the *gdpS* complementary strains were created with different mutated nucleotides ranging from the whole 18 nt to 4 nt as shown in Fig. 4A. We compared the transcript levels of *sarS* in these four complementary mutant strains with those of the wild-type strain and the *gdpS* mutant strains. Our real-time RT-PCR data indicated that expression of *sarS* in the four complementary mutant strains was as low as that in the mutant strain (Fig. 4B), suggesting that the regulation of *sarS* by *gdpS* is dependent on the putative 18-nt region. In addition, *lacZ* reporter assays were performed to demonstrate the significance of the 18-nt region for the interaction *in vivo* (see Fig. S2 in the supplemental material).

***gdpS* mRNA contributes to the stabilization of *sarS* mRNA.** *gdpS* mRNA appears to upregulate the transcript level of *sarS*, as the inactivation of *gdpS* decreases the transcript level of *sarS* mRNA. Therefore, we hypothesized that *gdpS* mRNA contributes to the stabilization of *sarS* mRNA by direct binding to *sarS* mRNA. To confirm this hypothesis, *sarS* mRNA half-life analysis was performed in the wild type, the *gdpS* mutant, and the complementary C/ATG-M strain. The equivalent cell aliquots were treated with rifampin. After that, total RNA was extracted, and the mRNA levels of *sarS* were determined by a real-time RT-PCR assay. The *sarS* mRNA level in the *gdpS* mutant reduced more rapidly than that in the wild type and the complementary strains (Fig. 5). And in the complementary strain, the half-life of *sarS* mRNA can be restored to a level as high as that in the wild type. These data indicated that *gdpS* mRNA upregulates *sarS* mRNA levels by extending the half-life of *sarS* mRNA.

***gdpS* mRNA cannot bind to *spa* mRNA *in vitro*.** Our previous work indicated that *gdpS* regulates the expression of *spa*. Thus, further experiments were carried out to determine whether *gdpS* mRNA directly interacts with *spa* mRNA. Sequence alignments indicate that no possible binding site exists between *gdpS* mRNA and the 5′UTR of *spa* mRNA. We then performed gel-shift assays to determine whether *gdpS* mRNA can bind to *spa* mRNA *in vitro*, with the reverse transcript of *gdpS* as the positive control. As shown in Fig. 6, *spa* mRNA cannot form a complex with biotin-labeled *gdpS* mRNA *in vitro*, suggesting that *gdpS* mRNA does not directly affect *spa* mRNA. These results suggest that *gdpS* regulates *spa* expression through *sarS*.

DISCUSSION

In *S. aureus* NCTC8325, *gdpS* is the only conserved GGDEF domain protein-encoding gene. Several reports have shown that GdpS in both *S. epidermidis* and *S. aureus* cannot synthesize c-di-GMP (19). Our previous work indicated that *gdpS* is involved in the virulence regulation, in particular, in the transcription of *spa* through *sarS* (20). However, the details of the regulatory mecha-

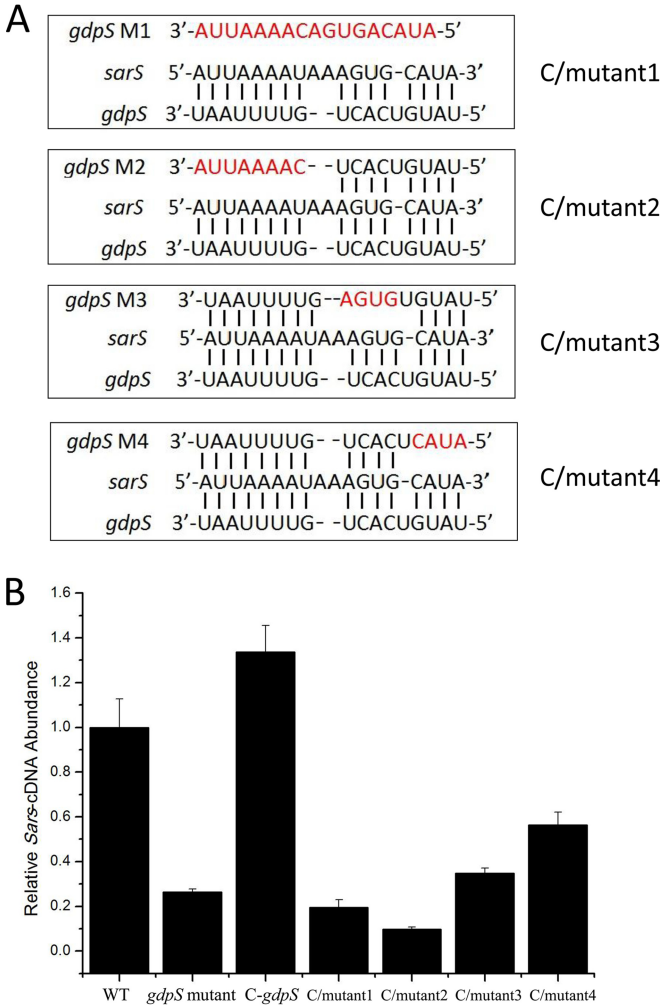

FIG 4 Regulation of *sarS* relies on the 18-nt binding site. (A) Maps of *gdpS* complementary plasmids with different site-directed mutations (C/mutant1, C/mutant2, C/mutant3, and C/mutant4) at the possible binding site. The mutated nucleotides are indicated in red and compared with the original sequence. (B) Real-time RT-PCR analysis of the *sarS* mRNA levels in the wild type, the *gdpS* mutant, and C-*gdpS* (the *gdpS* mutant with a plasmid containing whole *gdpS*), C/mutant1, C/mutant2, C/mutant3, and C/mutant4.

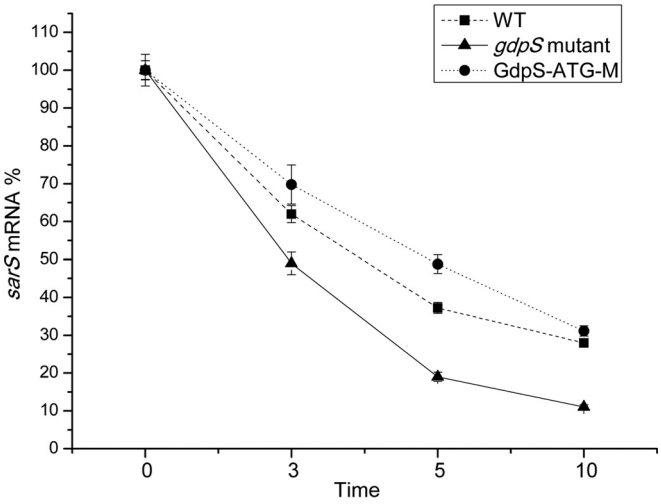

FIG 5 Analysis of mRNA half-life in the wild-type, the *gdpS* mutant, and C/ATG-M strains. The WT, the *gdpS* mutant, and C/ATG-M strains were treated with rifampin to block *de novo* RNA synthesis. Samples were taken for RNA isolation at 0, 3, 5, and 10 min after treatment and were analyzed by real-time RT-PCR. The data represent means of the results of three independent experiments.

nism of *gdpS* remained unclear. In this study, we have investigated the regulation of *sarS* and *spa* by *gdpS* in detail and found that *gdpS* mRNA can stabilize *sarS* mRNA by direct base pairing, resulting in the production of more SarS protein, which in turn upregulates *spa* expression (Fig. 7).

As an mRNA, *gdpS* mRNA has dual functions. *gdpS* mRNA not only can be routinely translated into protein but also can directly bind to the target mRNA to influence its stability, fulfilling the regulation of the target gene. Dual-function RNAs are not very common, and only a few examples have been reported (28). For example, *E. coli* RNA SgrS can regulate several mRNA targets through direct base pairing and encodes the 43-amino-acid polypeptide SgrT (29). The 39-amino-acid peptide SR1P, encoded by SR1 RNA, was also detected from a multicopy plasmid by Western blotting in *Bacillus subtilis* (30). In *Pseudomonas aeruginosa*, the Hfq-binding RNA PhrS contains a translated 37-amino-acid open reading frame (ORF) whose function is still unknown (31). In *S. aureus*, RNAIII is the only RNA reported to have dual functions, and its 3′-end domain is particularly important in base pairing with several mRNA targets (32–34); the 5′ end exhibits 75% complementarity to the 5′ untranslated region of the *hla* transcript and activates its translation (35, 36). In addition to the RNA regulatory role, RNAIII also functions as an mRNA encoding a 26-amino-acid peptide, δ-hemolysin, which directly interacts with the host cell membrane to cause cell lysis (37). In this work, we focused on the transcript form of *gdpS* and determined the function of *gdpS* with respect to *sarS* regulation via mRNA-mRNA direct interaction. Our Western blotting and complementary experiments confirmed that the translation product of *gdpS* has no function in the regulation of *sarS* and *spa*. This study indicated that, in *S. aureus*, *gdpS* mRNA is the second dual-function RNA, resembling RNAIII.

It is fascinating that binding of *gdpS* mRNA can contribute to the stabilization of *sarS* mRNA. The majority of known regulatory small RNAs (sRNAs), such as OxyS and RyhB RNA in *E. coli* and GcvB RNA in *Salmonella*, negatively regulate their target mRNAs

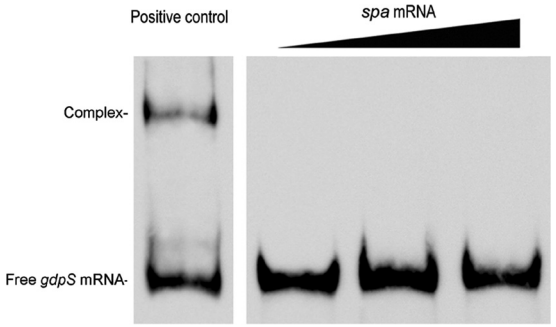

FIG 6 RNA-RNA gel-shift assays of *gdpS* mRNA and *spa* mRNA. Biotin-labeled *gdpS* mRNA probes were used in all reactions, and the antisense RNA was used as a positive control. Increasing amounts of *spa* mRNA were incubated with the biotin-labeled *gdpS* mRNA probes.

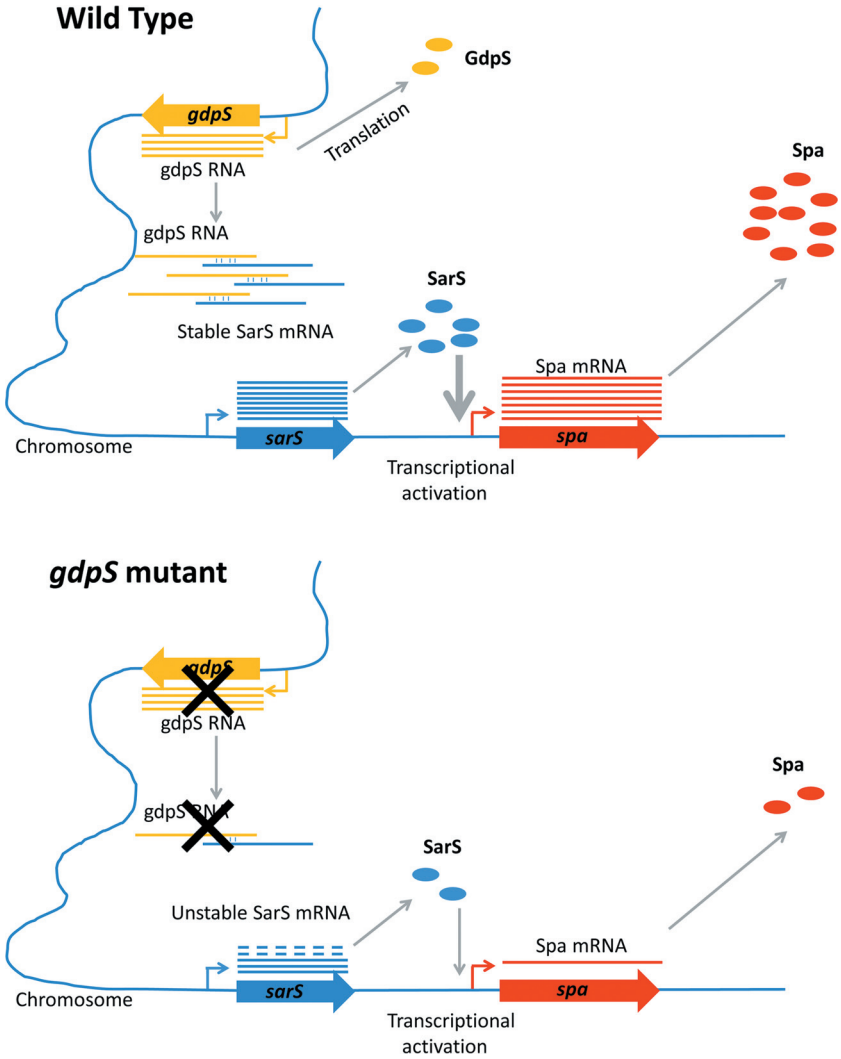

FIG 7 Proposed regulation of *spa* by *gdpS*. In *S. aureus*, *gdpS* mRNA upregulates the mRNA levels of *sarS* by binding to *sarS* mRNA, thus extending the half-life of *sarS* mRNA, which subsequently regulates the expression of *spa*.

through translation inhibition or through mRNA degradation (38–40). There are only a few examples of sRNAs that can positively regulate their target mRNAs. For example, GadY RNA confers increased stability of *gadX* mRNA through basing pairing with its 3′ untranslated region in *E. coli* (41). DsrA binding can disrupt the hairpin structure formed in the 5′UTR of *rpoS* mRNA and protects the mRNA from degradation by RNase E (42). According to the prediction of the RNA secondary structure by the mfold Web Server, the 5′UTR of *sarS* mRNA upstream of the translation initiation site forms a hairpin structure as well, which allows us to speculate that the binding between *gdpS* and *sarS* mRNA might stabilize *sarS* in a similar way.

The small RNA chaperone *hfq* plays an important role in sRNA binding in Gram-negative bacteria, while its function remains controversial in *S. aureus* (43). In this study, we also explored whether *hfq* plays a role in the regulatory process, and our real-time RT-PCR data (see Fig. S3 in the supplemental material) suggest that *hfq* is not involved in the regulation of *sarS* by *gdpS*.

In summary, we carried on the work begun in a previous study which showed that *gdpS* regulated the transcription of *spa* through *sarS* (20). This work reveals that the regulatory process is performed by the direct binding between *gdpS* and *sarS* mRNA and that the GdpS protein is not involved in the regulation. The identification of *gdpS* mRNA as a dual-function RNA in *S. aureus* might prompt the study of dual-function RNA in other organisms.

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