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The *Staphylococcus aureus* GGDEF Domain-Containing Protein, GdpS, Influences Protein A Gene Expression in a Cyclic Diguanylic Acid-Independent Manner▽

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***Staphylococcus aureus* is an important human pathogen that is the principal cause of a variety of diseases, ranging from localized skin infections to life-threatening systemic infections. The success of the organism as a pathogen and its ability to cause such a wide range of infections are due to its extensive virulence factors. In this study, we identified the role of the only GGDEF domain protein (GdpS [*G*GDEF *d*omain *p*rotein from *Staphylococcus*]) in the virulence of *S. aureus* NCTC8325. Inactivation of *gdpS* results in an alteration in the production of a range of virulence factors, such as serine and cysteine proteases, fibrinogen-binding proteins, and, specifically, protein A (Spa), a major surface protein of *S. aureus*. The transcript level of *spa* decreases eightfold in the *gdpS* mutant compared with the parental NCTC8325 strain. Furthermore, the transcript level of *sarS*, which encodes a direct positive regulator of *spa*, also decreases in the *gdpS* mutant compared with the wild type, while the transcript levels of *agr*, *sarA*, *sarT*, and *rot* display no apparent changes in the *gdpS* mutant, suggesting that GdpS affects the expression of *spa* through interaction with SarS by unknown mechanisms. Furthermore, the complementation assays show that the influences of GdpS on *spa* and *sarS* depend on its N-terminal domain, which is predicted to be the sensor of a two-component system, rather than its C-terminal GGDEF domain with conserved GGDEF, suggesting that GdpS functions in *S. aureus* by an unknown mechanism independent of 3′,5′-cyclic diguanylic acid signaling.**

Staphylococcus aureus is a well-known human pathogen which is the most common cause of a broad range of infections in humans involving all organ systems, ranging from localized skin infections to life-threatening systemic infections (3, 30). Production of a wide range of virulence factors is thought to be a key to this organism's ability to colonize, infect, and eventually cause disease in its host tissue (4, 27). These factors include secreted proteins, such as serine and cysteine proteases, nuclease, hemolysins, enterotoxins, lipase, and coagulase, and proteins exposed on the cell surface, such as protein A (Spa) and fibrinogen-, fibronectin-, and collagen-binding proteins (14, 34). Most of the studies of the mechanisms of staphylococcal pathogenesis have focused mainly on the regulatory mechanisms involved in the virulence factor gene expression in order to institute a more efficient infection control model (4, 10, 33).

Among the many virulence factors, Spa is a cell wall-associated exoprotein that binds to the Fc regions of immunoglobulin Gs (IgG) of diverse mammalian species and is thought to be an important component of the immune evasion machinery of this pathogen (16, 21, 29, 45). Previous studies have shown that strains of *S. aureus* with a high Spa content are more resistant to phagocytosis by human neutrophils in vitro than strains with smaller amount of Spa, possibly owing to the IgG Fc-binding property of Spa. The role of Spa in the pathogenesis of staphylococcal infections has been investigated in animal models. In a murine septic arthritis model, the wild-type strain was observed to produce a more severe disease condition than the Spa-deficient strain, indicating that protein A is an important virulence factor in arthritis (36, 38).

spa expression has been suggested to be regulated in a complicated way by a variety of factors (9, 20, 37). Among these factors, the first regulatory component identified was Agr, which is a repressor of *spa* transcription (22). The Agr system generates two divergent transcripts, RNAII and RNAIII, and RNAIII has been identified to be responsible for the downregulation of *spa* expression, not only at the transcriptional level but also by the RNAIII-mediated inhibition of translation and degradation of the stable *spa* mRNA by RNase III (24, 50). Expression of *spa* is also controlled by SarA. Like Agr, SarA also represses the transcription of the *spa* gene (9). Recently, additional regulatory components, including the SarA homologs SarS, SarT, and Rot, have been confirmed to be associated with this regulation (11, 41, 42, 48). Interestingly, it has been revealed that SarS, which is encoded by a gene located immediately upstream of *spa*, appears to be a key regulator in this regulatory network and to be responsible for the Agr- and SarA-dependent repression of protein A synthesis (11, 48).

In recent years, the 3′,5′-cyclic diguanylic acid (c-di-GMP)

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
S. aureus		
NCTC8325	Wild type	NARSA[a]
RN4220	8325-4, r^-	NARSA
SX3	8325 *gdpS*::*ermB*	This study
SX4	8325 *gdpS*::*ermB*(pLIgdpS)	This study
SX5	8325 *gdpS*::*ermB*(pLIlyt)	This study
SX6	8325 *gdpS*::*ermB*(pLIgdpSM)	This study
SX7	8325 *gdpS*::*ermB*(pLIgdpSM2)	This study
E. coli DH5α	Host strain for cloning	Laboratory stock
Plasmids		
pEASY-TB	Cloning vector; Kan^r Ap^r	TransGen
pEC1	pBluescript derivative; source of *ermB* gene; Ap^r	R. Bruckner
pBT2	Shuttle vector; temp sensitive; Ap^r Cm^r	R. Bruckner
pBTgdpS	pBT2 containing 380-bp upstream and 500-bp downstream fragments of *gdpS* and *ermB* genes; for *gdpS* mutagenesis; Ap^r Cm^r Em^r	This study
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
pLIgdpSM	pLIgdpS with a deletion mutation (GGEEF to EEF)	This study
pLIgdpSM2	pLIgdpS with a 51-bp deletion mutation in the N-terminal 5TMR-LYT domain region	This study

[a] NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*.

signaling system has drawn much attention. c-di-GMP was initially described as an allosteric activator of cellulose synthase in *Gluconacetobacter xylinus* and is now recognized as a second messenger ubiquitous in bacteria and involved in the regulation of a number of complex physiological processes (13, 26, 40, 46). This novel second messenger is synthesized by a class of enzymes containing GGDEF domains and hydrolyzed by EAL or HD-GYP domain proteins. Although the role of c-di-GMP as a second messenger has been extensively studied in diverse bacteria, its role in some low-GC gram-positive bacteria still remains obscure (5, 19, 27). The genome (CP000253) of *S. aureus* NCTC8325 encodes only one GGDEF domain-containing protein and another protein with a modified GGDEF domain, whereas no EAL domain-containing proteins are encoded. A recent study focusing on the GdpS protein in *Staphylococcus epidermidis* showed that inactivation of *gdpS* resulted in impaired biofilm formation capacity, and this function was independent of c-di-GMP signaling. In addition, in vitro study demonstrated that the GdpS proteins in both *S. epidermidis* and *S. aureus* cannot synthesize c-di-GMP. Therefore, the authors suggested that staphylococci may have only remnants of the c-di-GMP signaling pathway (23).

In this study, we investigated the function of the *S. aureus* NCTC8325 GdpS protein, which comprises the N-terminal sensor domain and the C-terminal GGDEF domain. We identified the transcriptional profiling affected by GdpS by using microarray analysis, and we carried out further study of its influence on the virulence of *S. aureus*, especially on *spa* expression. Our data indicated that GdpS affects *spa* transcription through SarS but not Agr, SarA, SarT, or Rot. In addition, consistent with the previous work on GdpS in *S. epidermidis*, the GdpS protein in *S. aureus* was observed to function similarly, depending on the N-terminal domain rather than the C-terminal GGDEF domain.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α was grown in Luria-Bertani (LB) medium, while the plasmid-containing *E. coli* strains were grown in the same medium but with added antibiotics (ampicillin, 100 mg/liter; kanamycin, 50 mg/liter). *Staphylococcus aureus* and its derivative strains were grown in tryptic soy broth (TSB) (soybean-casein digest medium USP; Oxoid) medium, and when necessary, erythromycin (2.5 mg/liter) and chloramphenicol (15 mg/liter) were added. The media were solidified with 1.5% (wt/vol) agar if needed.

DNA manipulation. Genomic DNA of *S. aureus* NCTC8325 was prepared using a standard protocol for gram-positive bacteria (15). Plasmid DNA from *E. coli* was extracted using a plasmid purification kit (Promega) according to the manufacturer's instructions. Plasmid DNA from *S. aureus* was extracted using the same kit, except that the cells were incubated for at least 30 min at 37°C in the solution of lysostaphin (Sigma) before the extraction process. The *Taq* and *Pfu* DNA polymerases were obtained from Promega, and the Primer Star DNA polymerase was obtained from Takara. Restriction enzymes were obtained from New England BioLabs, and the incubation conditions were as recommended by the suppliers. *Staphylococcus aureus* was transformed by electroporation as described previously (28).

Construction of the *S. aureus gdpS* mutant strain. To construct the deletion mutants, a 380-bp fragment that flanked the upstream region of the *gdpS* (SA OUHSC_00760) sequence and a 500-bp fragment that flanked the downstream region of the *gdpS* sequence were amplified by PCR with, respectively, the primers up-gdp-f-EcoRI, up-gdp-r-XbaI, down-gdp-f-XhoI, and down-gdp-r-SalI, using chromosomal DNA from *S. aureus* NCTC8325 as the template. A 1.5-kb erythromycin resistance gene was PCR amplified using primers Em-f-XbaI and Em-r-XhoI from pEC1 (6). The three fragments were mixed and digested by XbaI and XhoI, and then the digestions were purified and ligated. Using the ligation product as the template, 2.4-kb fragments were amplified by PCR with the primers up-gdp-f-EcoRI and down-gdp-r-SalI. The 2.4-kb fragments were then digested by EcoRI and SalI and cloned into the EcoRI/SalI sites of the shuttle plasmid pBT2 to create pBTgdpS. Allelic replacement of the native

TABLE 2. Oligonucleotide primers used in this study

Primer name	Oligonucleotide[a]
Up-gdp-f-EcoRI	GCGGAATTCGCAGTTGAACTGACAAAACTATATG
up-gdp-r-XbaI	GCGTCTAGAACAACGACAAAACTGTTAATCCTTTG
down-gdp-f-XhoI	GCGCTCGAGGTTAAAATGTTGCCTTAAAT
down-gdp-r-SalI	GAGGTCGACTAAGAAATCCAAAATGAATCGTTGT
Em-f-XbaI	GCGTCTAGAGATACAAATTCCCCGTAGGC
Em-r-XhoI	GCGCTCGAGGAAATAGATTTAAAAATTTCGC
c-gdpS-f-EcoRI	GAGGAATTCGTCGTTGTTATAAATTGATAATAGG
c-gdpS-r-BamHI	GAGGGATCCGGCAACATTTTAACATCAAA
c-lyt-f-EcoRI	GCGGAATTCTTAATGCCAAATATCGACAAATGTT
m-sagdpS-f	ATTTCTAAAAATTTTAAATTGATTCGG
m-sagdpS-r	GAAGAGTTCTCAGTTGTCATTCACA
m2-sagdpS-f	TACGATTGTCATTAAAACAGTGAC
m2-sagdpS-r	CACTTAACATTCGTTCCTTTATTG
RT-sarS-f	TTCAATATCTGAAGAACAACGAG
RT-sarS-r	TGAGGGTATTTATGGTGGATT
RT-spa-f	AAGATGGTAACGGAGTACATGTCG
RT-spa-r	TAATAACGCTGCACCTAAGGCTAA
RT-sarA-f	GACATACATCAGCGAAAA
RT-sarA-r	TACGTTGTTGTGCATTAA
RT-agrA-f	AAAGTTGCAGCGATGGATTT
RT-agrA-r	ATGGGCAATGAGTCTGTGAG
RT-RNAIII-f	GGTTATTAAGTTGGGATGG
RT-RNAIII-r	GAGTGATTTCAATGGCACA
RT-sspA-f	AATGTGGGAAAGTAAAGGAA
RT-sspA-r	ATCTGGGTTATTAGGTTGGT
RT-sspB-f	CTTGTATCGCTTCGTTTT
RT-sspB-r	TAGACCAAATTAAAGATA
RT-sdrD-f	AGATGGTAAGCAGGATT
RT-sdrD-r	AGTTGTAAGTCGGTTTG
RT-rot-f	CACTTTTGGGTGACATTA
RT-rot-r	CTTTCATCGTCAACAGGA
RT-sarT-f	AGAGTATTAACTACATATGAGCTG
RT-sarT-r	CATTTATTCAAGTAACCCTT
RT-16S-f	CGTGGAGGGTCATTGGA
RT-16S-r	CGTTTACGGCGTGGACTA

[a] Underlining indicates restriction endonuclease recognition sites.

gdpS gene with the resulting plasmid in the genomic DNA of *S. aureus* NCTC8325 with Em^r was carried out as described previously (6). Erythromycin-resistant and chloramphenicol-sensitive colonies were screened. PCR and sequencing were out to confirm that the desired gene inactivation had occurred by double-crossovercarried recombination. The sequences of all primers used in this study are listed in Table 2.

Complementation of the *gdpS* mutant. The *gdpS* gene and its promoter from *S. aureus* NCTC8325 were amplified by PCR with primers c-gdpS-f-EcoRI and c-gdpS-r-BamHI, while the *gdpS* promoter and its N-terminal 5TMR-LYT domain were amplified by PCR with primers c-lyt-f-EcoRI and c-gdpS-r-BamHI. The PCR products were, respectively, cloned into pLI50 to create plasmids pLIgdpS and pLIlyt. The method of site-directed mutagenesis by PCR was used to mutate the conserved GGEEF motif or the 5TMR-LYT domain of the GdpS protein. For example, to create the plasmid pLIgdpSM, the plasmid pLIgdpS was used as a template for the PCRs, and the primers for constructing a deletion mutation (GGEEF to EEF) were m-sagdpS-f and m-sagdpS-r. A DNA fragment (6,678 bp, containing the full length of pLIgdpS except the 6-bp nucleotide bases) was amplified by PCR with Primer Star DNA polymerase. The PCR products were digested with DpnI to remove the template plasmids, and subsequently, the digested products were phosphorylated, self-ligated, and transformed into *E. coli* DH5α. The positive clones with mutational plasmids were verified by DNA sequencing, yielding pLIgdpSM. The four plasmids were transformed by electroporation into *S. aureus* RN4220 and subsequently transferred to strain *S. aureus* NCTC8325 *gdpS*::*ermB*.

Biofilm and autolysis assays. Semiquantitative measurements of biofilm formation under static conditions were done using Costar 3599 96-well plates (Corning), as described previously (12, 44). Triton X-100-stimulated autolysis was measured as described previously (43). The cells were grown in PYK medium (5.0 g of Bacto Peptone, 5.0 g of yeast extract, and 3.0 g of K_2HPO_4 per liter at pH 7.2) to mid-exponential phase at 30°C with constant shaking (200 rpm). After centrifugation, the cells were washed with cold double-distilled water, resuspended in 0.05 M Tris-HCl (pH 7.5) buffer containing 0.05% (wt/vol) Triton X-100 in spectrophotometer vials, and incubated at 30°C with constant shaking (200 rpm). The decrease in the optical density at 580 nm (OD_{580}) was measured every 30 min.

Proteolytic activities and fibronectin binding assays. Proteolytic activities were determined using the insoluble proteolytic substrate azocoll (Sigma) as previously described (17), with some modifications. The substrate (4 mg/ml) was suspended in 100 mM phosphate buffer (pH 7.0). *Staphylococcus aureus* strains were grown in TSB for 12 h, and every strain was grown to the same OD. About 400 to 500 μl of culture supernatant was added to 400 μl of the substrate suspension. The mixtures were incubated for 4 h at 37°C with constant shaking, and the reaction was stopped by removing the substrate by centrifugation. An aliquot was removed, and the absorbance was measured at 530 nm.

The fibronectin binding assay was essentially a modification of that used by Ahmed et al. (1). Costar 3590 96-well plates (Corning) were coated with 100 μl of 0.02% sodium carbonate (pH 9.6) containing fibronectin (10 mg/ml; Sigma) overnight at 4°C and then blocked with 100 μl of 2% bovine serum albumin solution for 1 h at 37°C. The wells were washed thrice with 100 μl of phosphate-buffered saline. Subsequently, 100 μl of bacteria (corresponding to 10^6, 10^7, or 10^8 cells) was added, in quadruplicate, to the appropriate wells and incubated for 2 h at 37°C. After that, the bacteria were fixed with 100 μl of 25% formaldehyde for 10 min. Subsequently, 100 μl of 0.5% crystal violet was added to each well and left for 1 min, and the absorbance was measured at 570 nm.

Total RNA isolation, cDNA generation, and microarray processing. Total RNA was isolated using RNeasy Mini kits (Promega) according to the manufacturer's instructions. The cDNA was synthesized and labeled according to the manufacturer's suggestions for *S. aureus* antisense genome arrays (Affymetrix Inc., Santa Clara, CA). Further preparation, hybridization, and scanning were carried out by Biochip Company of Shanghai. Real-time reverse transcription-PCR (RT-PCR) was also performed as previously reported (51), using an Applied Biosystems 7000 real-time PCR system (Applied Biosystems). The 16S rRNA was used for normalizing all the reactions, and its transcript levels showed minimal variation between wild-type and mutant cells (data not shown). Microarray data were analyzed with the Affymetrix Microarray Suite software 5.1 (Affymetrix Inc.) and a four-comparison survival method (8).

Western blot analysis of Spa. Western blot analysis was performed using a modified method as previously described (18). The cells were harvested at an OD_{600} of 1.5, and the cell wall-associated proteins were released from the bacterial cells by lysis after incubation with lysostaphin (24 U/ml) and were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel and electroblotted onto a polyvinylidene difluoride membrane (Hybond; GE). The membranes were incubated with anti-Spa rabbit IgG (Sigma), and the IgG bound to Spa was detected using horseradish peroxidase-conjugated sheep anti-rabbit antibodies (Pierce).

Microarray data accession number. The microarray data have been submitted to the CIBEX database (http://cibex.nig.ac.jp) with the accession number CBX74.

RESULTS

Bioinformatic analysis of GdpS in *S. aureus*. The available information on the *S. aureus* genome shows that *gdpS* (SAOUHSC_00760) is an independent open reading frame (Fig. 1A). Upstream of *gdpS*, there exists an operon predicted to be involved in glutathione metabolism. A gene called *llm* is located downstream of *gdpS*, encoding a lipophilic protein that affects the lysis rate and methicillin (meticillin) resistance level (31). The *gdpS* encodes a protein containing two domains: the N-terminal 5TMR-LYT domain and the C-terminal conserved GGDEF domain (Fig. 1B). The N terminus of GdpS contains several transmembrane regions, five of which form a 5TMR-LYT domain, which has been proposed to be the sensor of the LytS-YhcK-type histidine protein kinase. The histidine kinase LytS affects autolysis and is involved in the regulation of murein hydrolases (2, 7, 32). The C-terminal GGDEF domain of GdpS contains most of the motifs and residues involved in GTP binding. However, we observed several nonhomologous residues between the GdpS GGDEF domain and the invariable residues of the GGDEF domain consensus sequence, which is consistent with previous findings (23). The study of the GdpS protein in staphylococci has indicated that the GdpS proteins in both *S. aureus* and *S. epidermidis*, which share

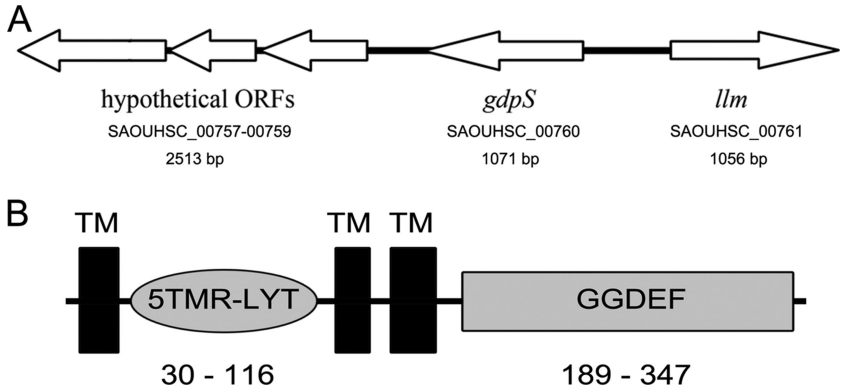

FIG. 1. Bioinformatics identification of *gdpS* (SAOUHSC_00760) in *S. aureus* NCTC8325. (A) Chromosomal organization of the *gdpS* gene and its surrounding region. Upstream of *gdpS*, there exists an operon predicted to be involved in glutathione metabolism. Downstream of *gdpS*, there exists a gene divergently transcribed from *gdpS*, encoding a lipophilic protein that affects the lysis rate and methicillin resistance level. (B) Predicted domain structure of GdpS. TM, transmembrane domain. 5TMR-LYT is a transmembrane domain proposed to be the sensor of the LytS-YhcK-type histidine protein kinase.

82.0% similarity at the amino acid level, have only GTP-binding ability and not c-di-GMP synthetic activity (23).

Phenotypic assays of the *gdpS* mutant strain. To ascertain whether GdpS affects the growth of *S. aureus*, we assessed the growth rates of the mutant strain and the parental strain, and our results showed no remarkable difference in the growth of the two strains when they were grown in LB or TSB medium (data not shown). Previous reports have suggested that the *lytS* mutant of *S. aureus* exhibits increased autolysis and a marked propensity to form aggregates in liquid culture (7). Our results showed that the *gdpS* mutant exhibited almost the same rate of autolysis as the parental strain (data not shown), suggesting that the N terminus of GdpS might not function similarly to the general LytR-LytS two-component system in *S. aureus*. We also performed biofilm assays. Although a previous study indicated that GdpS influenced biofilm formation in *S. epidermidis* when the cells were grown in brain heart infusion (BHI) medium supplemented with 4% NaCl (23), the *gdpS* mutant of *S. aureus* NCTC8325 showed the same biofilm formation capacity as the wild type when grown in TSB, BHI medium, or BHI medium supplemented with 4% NaCl (data not shown). In addition, we performed phenotypic assays to monitor the protease activity and fibronectin binding of both the *gdpS* mutant and the wild type. The total protease activity in the culture supernatant of the *gdpS* mutant increased by >60% relative to that for the wild type, as determined with azocoll, whereas the *gdpS* mutant demonstrated a level of adherence to fibronectin similar to that of the wild type (data not shown). To further investigate how *gdpS* deletion affects the cellular activities, we performed microarray analysis of these two strains.

Effect of GdpS on *S. aureus* gene transcription. To characterize the gene transcriptional profiling influenced by GdpS, DNA microarray assays were performed using the parental strain NCTC8325 and the *gdpS* deletion mutant strain. The cells were grown in TSB medium to an OD_{600} of 1.7. A twofold induction ratio was used as a cutoff limit to compare the transcriptional profiling in the wild type and the *gdpS* mutant strain. Microarray data indicated that 77 genes were induced and 47 genes were repressed in the *gdpS* mutant strain. Among these genes, several major classes were associated with metabolism, signal transduction, and virulence of *S. aureus* (Table 3). The transcript levels of a range of virulence factors were altered in the *gdpS* mutant strain, such as *sdrC*/*sdrD* (encoding Ser-Asp-rich fibrinogen-binding and bone sialoprotein-binding protein), *sspA*/*sspB*/*sspC* (encoding extracellular proteases), and particularly *spa* (encoding the IgG-binding protein A precursor), whose transcript level decreased eightfold in the *gdpS* mutant strain. Interestingly, the transcript level of *sarS*, which has been demonstrated to be involved in the positive regulation of *spa* transcription by directly binding to the *spa* promoter, decreased about threefold in the mutant strain compared with the parental strain, suggesting that GdpS might influence *spa* transcription via SarS. Furthermore, the transcript levels of a set of selected genes were verified with real-time RT-PCR measurements. Figure 2 shows that there was a positive correlation between the two techniques.

GdpS affects Spa at the transcription level via a SarS regulating pathway. According to our DNA microarray data, the transcript level of *spa* decreased about eightfold in the *gdpS* mutant strain compared with the parental strain. *spa* has been suggested to be regulated by a range of factors, including Agr, SarA, SarS, SarT, and Rot. In the Agr system, RNAIII has been suggested to repress *spa* expression. As RNAIII is a small RNA whose expression information was not reflected in the microarray assay, we performed real-time RT-PCR to determine if the change in *spa* transcription was associated with RNAIII repression. The transcript levels of *agrA*, *sarA*, *sarS*, *sarT*, and *rot* in the wild type and the *gdpS* mutant were also compared using real-time RT-PCR analysis. Interestingly, the transcript levels of *agr*, *sarA*, *sarT*, and *rot* displayed no apparent alteration in *gdpS* mutant compared with the parental strain. In contrast, the expression of *sarS* was influenced by GdpS (Fig. 3). As mentioned above, the microarray data showed that the transcript level of *sarS* decreased in the *gdpS* mutant, and our real-time RT-PCR indicated that GdpS indeed repressed the transcription of *sarS*. Previous studies have shown that the expression of *spa* was lower in the *sarS* mutant than in the parental strain, *S. aureus* NCTC8325-4 (11, 35). To further evaluate whether GdpS affects Spa via SarS, we attempted to complement the *gdpS* mutant with a plasmid con-

TABLE 3. Main genes affected by GdpS

Open reading frame	Gene	Gene product	Log_2 ratio (wild type/mutant)
SAOUHSC_00974		Glycosyl transferase, group 1 family protein	1.8
SAOUHSC_02057		dUTP pyrophosphatase	1
SAOUHSC_00069	*spa*	IgG-binding protein A precursor	2.7
SAOUHSC_00070	*sarH1/ sarS*	Staphylococcal accessory regulator A homolog	1.5
SAOUHSC_01164	*pyrR*	Pyrimidine regulatory protein PyrR	1
SAOUHSC_01165	*pyrP*	Uracil permease	2
SAOUHSC_01166	*pyrB*	Aspartate carbamoyltransferase catalytic subunit	1.6
SAOUHSC_01168	*pyrC*	Dihydroorotase	1.4
SAOUHSC_01169	*pyrAA*	Carbamoyl-phosphate synthase small subunit	1.6
SAOUHSC_01170	*carB*	Carbamoyl-phosphate synthase large subunit	1.5
SAOUHSC_01171	*pyrF*	Orotidine 5′-phosphate decarboxylase	1.3
SAOUHSC_01172	*pyrE*	Orotate phosphoribosyltransferase	1
SAOUHSC_01249	*ribA*	Riboflavin biosynthesis protein	1.1
SAOUHSC_01249	*ribB*	Riboflavin synthase subunit alpha	1.1
SAOUHSC_02606		Imidazolonepropionase	1.3
SAOUHSC_02607		Urocanate hydratase	1.8
SAOUHSC_00132	*gbsA*	Glycine betaine aldehyde dehydrogenase	2.2
SAOUHSC_02444	*cudT*	Osmoprotectant transporter, BCCT family, choline transporter	1.8
SAOUHSC_01013	*purL*	Phosphoribosylformylglycinamidine synthetase	1.8
SAOUHSC_00804	*smpB*	SsrA-binding protein	1.1
SAOUHSC_02932		Choline dehydrogenase	1.8
SAOUHSC_00544	*sdrC*	Ser-Asp-rich fibrinogen-binding, bone sialoprotein-binding protein	1.7
SAOUHSC_00545	*sdrD*	Ser-Asp rich fibrinogen-binding, bone sialoprotein-binding protein	2.1
SAOUHSC_00974		Glycosyl transferase, group 1 family protein	2.5
SAOUHSC_02820		ABC transporter, ATP-binding protein	1.2
SAOUHSC_01953	*epiA*	Lantibiotic epidermin precursor	−1.4
SAOUHSC_01939	*splC*	Serine protease	−1.2
SAOUHSC_00009		Seryl-tRNA synthetase	−1
SAOUHSC_02552		Similar to biotin biosynthesis protein	−1.3
SAOUHSC_02558		Urease gamma subunit	−1.1
SAOUHSC_02561		Urease alpha subunit	−1
SAOUHSC_01990		Glutamate ABC transporter ATP-binding protein	−1.5
SAOUHSC_02711	*bioX*	BioX protein, putative	−1.2
SAOUHSC_02712		6-Carboxyhexanoate-coenzyme A ligase	−1.2
SAOUHSC_02713		Similar to 8-amino-7-oxononanoate synthase	−1.6
SAOUHSC_02714	*bioB*	Biotin synthase	−2.1
SAOUHSC_02715	*bioA*	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase	−3.3
SAOUHSC_02716	*bioD*	Dethiobiotin synthetase	−4.2
SAOUHSC_02717		Sorbitol dehydrogenase homolog	−1
SAOUHSC_00986	*sspC*	Cysteine protease	−1.7
SAOUHSC_00988	*sspA*	Serine protease	−1.6
SAOUHSC_00987	*sspB*	Cysteine protease precursor	−1.3
SAOUHSC_02866		MmpL efflux pump, putative	−1.1
SAOUHSC_02883	*ssaA*	Staphyloxanthin biosynthesis protein, similar to secretory antigen precursor SsaA	−1
SAOUHSC_02971	*aur*	Zinc metalloproteinase aureolysin	−2.1
SAOUHSC_00317	*glpT*	Glycerol-3-phosphate transporter	−1
SAOUHSC_00557	*vraA*	Substrate-coenzyme A ligase, putative	−1
SAOUHSC_00627		Na antiporter, MnhC component, putative	−1.1
SAOUHSC_00831		Similar to general stress protein, OsmC/Ohr family protein	−1.2
SAOUHSC_00898	*argH*	Argininosuccinate lyase	−3
SAOUHSC_00899	*argG*	Argininosuccinate synthase	−3.7
SAOUHSC_00762	*llm*	Glycosyl transferase, group 4 family protein	−1.3

taining the full-length *gdpS*. As shown in Fig. 3, the transcript levels of *spa* and *sarS* in the *gdpS* mutant were complemented almost to the same extent as those in the wild type by the full-length *gdpS*.

To further demonstrate that the *gdpS* gene influences *spa* expression, we carried out Western blot analysis. The results showed that the expression level of Spa was higher in the wild type than in the *gdpS* mutant at an OD_{600} of 1.5 (Fig. 4B).

The GdpS effect on *spa* depends on the N-terminal domain. The previous report focusing on GdpS in *S. epidermidis* demonstrated that GdpS functions independent of the GGDEF domain (23). To investigate whether the effect of GdpS on *spa* expression was also independent of the C-terminal GGDEF domain in *S. aureus*, we conducted several types of complementation experiments. Four kinds of plasmids encoding the sequences of the whole GdpS, the N-terminal domain, GdpS with a mutated GGDEF domain (GGEEF to EEF), and GdpS with a mutated N terminus were constructed and transformed into the *gdpS* mutant. The transcript levels of *sarS* and *spa* in the above-mentioned three strains were compared with those

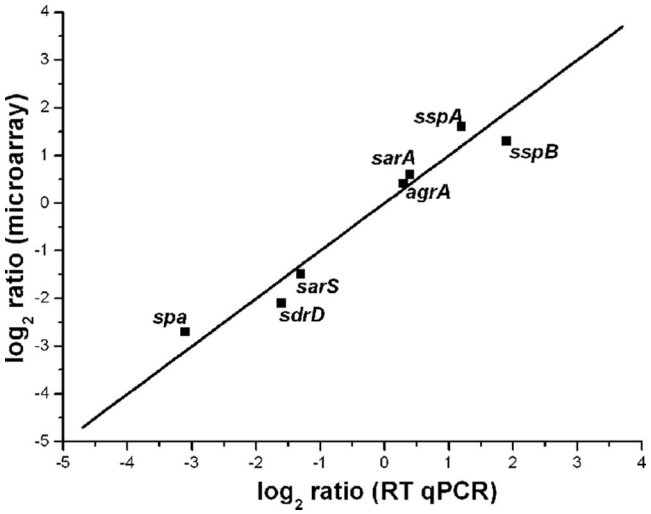

FIG. 2. Correlation of microarray and real-time RT-PCR results (microarray versus real-time RT-PCR). The differences in the transcription of seven genes were $\log_2$ transformed and plotted against each other. The diagonal line indicates that the ratio from the microarray data is similar as that from the real-time RT-PCR data. The abscissa represents the $\log_2$ ratio of mutant to wild type according to the real-time RT-PCR data, and the ordinate represents the $\log_2$ ratio of mutant to wild type according to the microarray data. The two methods are better correlated if the points are nearer the diagonal line.

in the wild-type strain using real-time RT-PCR. The results showed that the transcript levels of *spa* in strains SX4 (*gdpS* mutant with a plasmid encoding full-length GdpS), SX5 (*gdpS* mutant with a plasmid encoding the N terminus of GdpS), and SX6 (*gdpS* mutant with a plasmid encoding GdpS with mutated GGDEF domain) were complemented to nearly the same extent as that in the wild-type strain (Fig. 4A). However, in strain SX7 (*gdpS* mutant with a plasmid encoding GdpS with a mutated N terminus), the transcript levels of *spa* were still as low

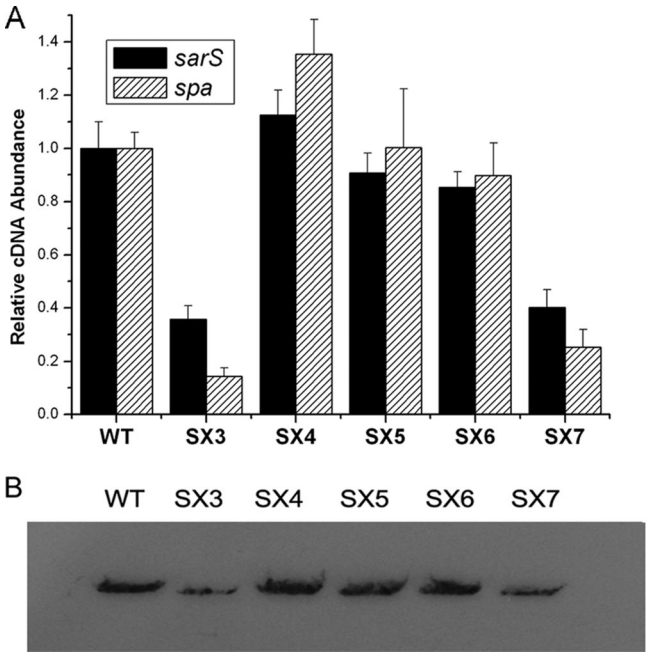

FIG. 4. (A) The transcriptional regulation of *spa* and *sarS* expression by GdpS was compared using real-time RT-PCR in *S. aureus* NCTC8325 (wild type [WT]), SX3 (*gdpS* mutant), SX4 (*gdpS* mutant with a plasmid encoding full-length GdpS), SX5 (*gdpS* mutant with a plasmid encoding the N terminus of GdpS), SX6 (*gdpS* mutant with a plasmid encoding GdpS with a mutated GGDEF domain), and SX7 (*gdpS* mutant with a plasmid encoding GdpS with a mutated N terminus). All the strains were grown in TSB medium to an OD_{600} of 1.7. The relative transcription of each gene compared to the constitutively expressed 16S rRNA gene in strains SX3, SX4, SX5, SX6, and SX7 was compared with that in the wild type, to which we assigned a value of 1. (B) Western blot analysis of Spa in *S. aureus* NCTC8325, SX3, SX4, SX5, SX6, and SX7. All the strains were grown in TSB medium to an OD_{600} of 1.5.

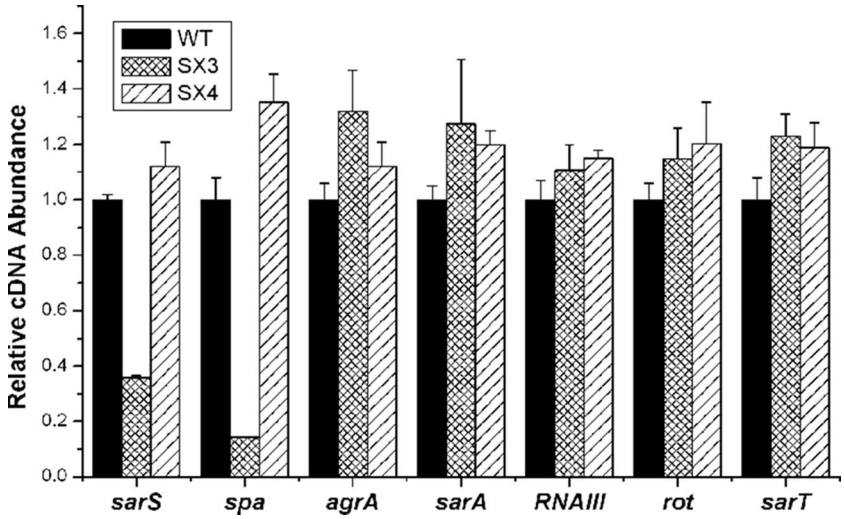

FIG. 3. Comparative measurement of *spa* and its regulator gene transcripts by real-time RT-PCR in *S. aureus* NCTC8325 (wild type [WT]), SX3 (*gdpS* mutant), and SX4 (*gdpS* mutant with a plasmid encoding full-length GdpS). All the strains were grown in TSB medium to an OD_{600} of 1.7. The relative transcription of each gene compared to the constitutively expressed 16S rRNA gene in SX3 and SX4 was compared with that in the wild type, to which we assigned a value of 1. Error bars indicate standard deviations.

as that in the mutant strain. Taking all this into account, it can be concluded that the effect of GdpS on *spa* relies on the N-terminal domain rather than the C-terminal domain.

DISCUSSION

Existing experimental evidence suggests that GGDEF/EAL are generally soluble cytoplasmic domains located C terminal to the often multiple sensory and signal transduction domains. A significant fraction of the GGDEF and EAL domains is linked to the cytoplasmic sensory domains involved in the binding of small-molecule ligands or in protein-protein interaction, while another sizable fraction is linked to the N-terminal periplasmic or integral membrane sensory domains, whose ligand-binding specificity is unknown (25, 39, 47). The N terminus of the *S. aureus* NCTC8325 GdpS protein is a transmembrane domain proposed as a sensor of the LytS-YhcK-type histidine protein kinase. Bioinformatic analysis demonstrated that LytS and YhcK, which share a conserved membrane-spanning domain with five transmembrane helices, are both parts of the signaling complexes that might be involved in cell wall metabolism. However, in this study, we found that the rates of autolysis in the *gdpS* mutant and the wild-type strain displayed no apparent difference, and accordingly, the microarray data indicated that no factors associated with autolysis and murein hydrolases were affected by GdpS. Our study suggested that the specific LytS-YhcK sensor domain might function based on a novel mechanism. Interestingly, the GdpS protein in *S. aureus* was found to be related to the expression of some virulence factors, including proteases and protein A.

We carried out a more detailed study of protein A, as it is a major determinant of the virulence of *S. aureus*. Previous studies of *spa* regulation in *S. aureus* revealed that the regulatory elements Agr/RNAIII and SarA both play important roles in repressing *spa* transcription not only through direct regulation but also by interacting with SarS (48). Expression of *sarS* is strongly repressed by *agr* and *sarA*. In contrast, SarT and Rot were observed to influence the expression of *spa* only though positive regulation of *sarS* expression (41, 42). According to our study, the *gdpS* deletion affected *spa* transcription and also altered the transcription of its regulator gene, *sarS*. However, the *gdpS* gene exhibited no influence on *agr*, *sarA*, *sarT*, and *rot*. From these results, we can conclude that GdpS affects the expression of *spa* via SarS in an RNAIII-, SarA-, SarT-, and Rot-independent manner (Fig. 5), although the detailed mechanism remains to be further explored.

As a regulator of virulence factor gene expression, SarS has been reported to negatively regulate serine protease and positively regulate the expression of several surface proteins (35). Both the microarray data and results of the phenotypic assays in this study showed that the protease activity was enhanced in the *gdpS* mutant, indicating that the expression of *ssp* might also be influenced by GdpS through SarS.

A previous study based on the screening of an *S. aureus* S30 transposon mutant library showed that the mutation of the *S. aureus gdpS* gene impaired the biofilm formation capacity (49). However, our physiological data revealed that the *gdpS* mutant displayed no difference in biofilm formation capacity compared to the wild type, which may possibly be due to some characteristic physiological differences between the strains. *S. aureus* strain S30 exhibited a stable and strong biofilm-forming phenotype on a variety of substrates and under various culture conditions (49), whereas the wild-type strain *S. aureus* NCTC8325 failed to exhibit strong biofilm formation under our experimental conditions.

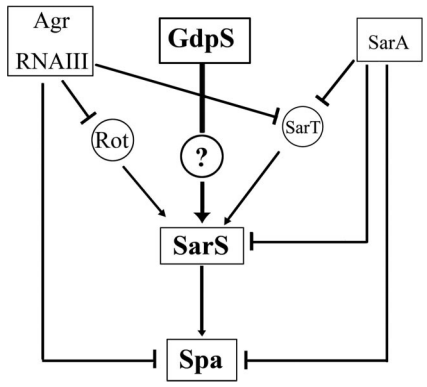

FIG. 5. Proposed regulation of Spa by GdpS, SarS, RNAIII, SarA, SarT, and Rot. Previous work has shown that expression of *spa* is negatively controlled by Agr/RNAIII and SarA through both direct regulation and interaction with SarS. SarT and Rot upregulate the expression of *spa* only though positive regulation of *sarS*. Our work indicates that GdpS influences expression of *spa* via SarS in an RNAIII-, SarA-, SarT-, and Rot-independent manner.

An earlier study of GdpS demonstrated that in *S. epidermidis* GdpS inactivation impaired the biofilm formation capacity in a c-di-GMP-independent manner and suggested that the GdpS proteins in staphylococci are only the remnants of the c-di-GMP signaling pathway as found in *Archaea* (23). Although GdpS is the only GGDEF domain-containing protein in *S. aureus* NCTC8325, our data further suggest that it might not function in c-di-GMP signaling.

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