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sarZ, a *sarA* Family Gene, Is Transcriptionally Activated by MgrA and Is Involved in the Regulation of Genes Encoding Exoproteins in *Staphylococcus aureus*[▽]

Anand Ballal, Binata Ray, and Adhar C. Manna*

Division of Basic Biomedical Sciences, University of South Dakota, Vermillion, South Dakota 57069, and Center for Infectious Disease Research and Vaccinology, South Dakota State University, Brookings, South Dakota 57007

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The expression of genes involved in the pathogenesis of *Staphylococcus aureus* is controlled by global regulatory loci, including two-component regulatory systems and transcriptional regulators (e.g., *sar* family genes). Most members of the SarA family have been partially characterized and shown to regulate a large numbers of target genes. Here, we describe the characterization of *sarZ*, a *sarA* paralog from *S. aureus*, and its regulatory relationship with other members of its family. Expression of *sarZ* was growth phase dependent with maximal expression in the early exponential phase of growth. Transcription of *sarZ* was reduced in an *mgrA* mutant and returned to a normal level in a complemented *mgrA* mutant strain, which suggests that *mgrA* acts as an activator of *sarZ* transcription. Purified MgrA protein bound to the *sarZ* promoter region, as determined by gel shift assays. Among the *sarA* family of genes analyzed, inactivation of *sarZ* increased *sarS* transcription, while it decreased *agr* transcription. The expression of potential target genes involved in virulence was evaluated in single and double mutants of *sarZ* with *mgrA*, *sarX*, and *agr*. Northern and zymogram analyses indicated that the *sarZ* gene product played a role in regulating several virulence genes, particularly those encoding exoproteins. Gel shift assays demonstrated nonspecific binding of purified SarZ protein to the promoter regions of the *sarZ*-regulated target genes. These results demonstrate the important role played by SarZ in controlling regulatory and virulence gene expression in *S. aureus*.

Staphylococcus aureus is a human pathogen that colonizes more than 1 billion people worldwide and causes a variety of infections, ranging from cutaneous infections (impetigo, folliculitis, and carbuncles) to deep-seated infections (pneumonia, endocarditis, septicemia, and osteomyelitis), and other metastatic complications (18, 21, 27). The primary site of infection is generally the skin or soft tissues, from which the organism can spread to the bloodstream and subsequently disseminate into various tissues. Once *S. aureus* establishes its presence in the tissue, it produces a large number of cell surface-associated components and secreted products that include adhesins, enzymes, toxins, capsular polysaccharides, and other gene products that facilitate tissue colonization, tissue destruction, or immune evasion. The expression of many of these genes is coordinately controlled by regulatory systems, such as such as *agr*, *arlRS*, *srrAB*, *saeRS*, *lytSR*, *vraRS*, *sigB*, *tcaRAB*, *sarA*, and nine other *sarA* paralogs (i.e., *sarR*, *sarS*, *sarT*, *sarU*, *sarV*, *rot*, *sarX*, *sarZ*, and *mgrA*) (2–23, 28–56).

The *agr* system is a well-studied regulatory system that controls the synthesis of select cell surface proteins and exoproteins. The *agr* locus consists of two divergent transcriptional units, *agr* RNAII and RNAIII. RNAII encodes the sensor kinase (AgrC), response regulator (AgrA), a signal-processing protein (AgrB), and an autoinducing peptide (AIP, or AgrD). RNAIII is a 514-nucleotide RNA regulatory molecule that regulates a large number of genes, mostly at the posttranscriptional level (23, 39–41). Microarray analysis in strain RN6390 showed that 104 and 34 genes are upregulated and downregulated, respectively, due to *agr* inactivation (13), suggesting that *agr* is a global regulator in *S. aureus*. Several reports have shown rather contradictory roles of the *agr* locus in the regulation of target genes, but these conflicting results could be due to the use of different strains, growth media, and growth conditions (5, 44). Besides the autoactivation of *agr* by AIP and AgrA, SarA and several of the SarA paralogs, *srrAB*, *arlSR* have also been shown to modulate RNAII and RNAIII transcription (2, 3, 10, 12, 15, 20, 34, 35, 39–41, 43, 45).

The *sarA* locus is known to upregulate the synthesis of fibronectin and fibrinogen binding proteins, hemolysins (α, β, and δ), enterotoxins, TSST-1 toxin, and capsule biosynthesis genes and to downregulate proteases, protein A, and a collagen binding protein. SarA also binds to several regulatory and target gene promoters (e.g., *agr*, *sarS*, *rot*, *sarV*, *sarT*, *hla*, *fnb*, *spa*, *cna*, *bap*, and *icaRA*) to modulate gene transcription, thus implicating both *agr*-dependent and *agr*-independent pathways in SarA-mediated regulation (2, 8–12, 33, 35, 36, 45, 49, 53). Using affinity chromatography and genome sequence information, nine SarA paralogs (i.e., SarR, SarS, SarT, SarU, SarV, SarX, SarZ, Rot, and MgrA) that are involved directly or indirectly in the regulation of target genes implicated in regulation, virulence, biofilm formation, autolysis, antibiotic resistance, and metabolic processes have been identified (10). SarS (also called SarH1), a 250-residue protein, is involved in activation of *spa* expression (9, 42, 52). Protein A, associated with the cell surface, is an important virulence factor involved in a wide variety of interactions with various host factors during

* Corresponding author. Mailing address: Division of Basic Biomedical Sciences, Sanford School of Medicine, University of South Dakota, 414 E. Clark Street, Vermillion, SD 57069. Phone: (605) 677-6336. Fax: (605) 677-6381. E-mail: amanna@usd.edu.

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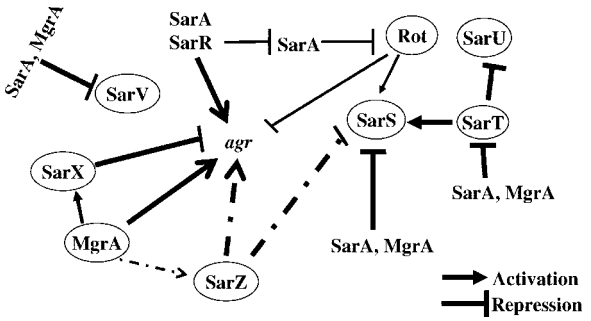

FIG. 1. Overview of the predicted intertwined regulatory networks of 10 *sar* family genes and their relationship with the *agr* locus. This overview is mostly based on genetic and biochemical analyses in strain RN6390 under normal conditions of growth (8, 10, 28–36, 41, 49–52). Several members of the *sar* family of genes (e.g., *sarV*, *sarT*, and *sarU*) are not transcribed due to repression by other normally expressed *sar* family genes (32, 33, 49). The pathways identified in this study are depicted by dashed lines. The thickness of the line indicates the nature of regulation: thin, partially involved, and thick, completely involved.

staphylococcal infection (41, 42). Inactivation of *mgrA*, shown to be involved in the regulation of virulence genes, as well as autolytic genes (11, 28, 29, 54), is also involved in oxidative-stress responses and indirectly affects resistance to antibiotics by controlling the expression of at least four efflux pumps (i.e., *norA*, *norB*, *norC*, and *tet38*) by repressing the expression of *norG* (6, 55). Previous studies have shown the SarA paralogs to be activated or repressed by other members of their own family. For example, MgrA positively and negatively regulates the expression of *sarX* and *sarV* transcripts, respectively, while SarT represses *sarU* transcription (32–34). The interregulatory relationship between different members of the SarA family is shown in Fig. 1. More recently, SarZ has been shown to be involved in the regulation of hemolysin production, and *sarZ* mutants are less virulent in silkworm and mouse infection models (20). Along with hemolysin (*hla* and *hlb*) transcripts, the *sarZ* mutants also showed reduced *agr* expression. It has also been demonstrated that the DNA binding ability of SarZ is essential for virulence in *S. aureus* (20). In a more recent study, *sarZ* has been shown to be important for biofilm formation and virulence in both *Staphylococcus epidermidis* and *S. aureus* (51, 56). Very recently, it has been shown that *sarZ* is involved in the activation of *mgrA* and the *agr* RNAIII molecule, and *S. aureus* with *sarZ* deleted showed increased transcription of *spa*, the protein A gene, while the levels of *hla* (the hemolysin gene) and *sspA* (the V8 protease gene) transcripts were decreased (51). A different study also suggested that SarZ is involved in oxidative sensing and regulation of genes involved in oxidative pathways (7).

In this report, we show that among the nine *sarA* paralog, *agr*, *sigB*, and *saeRS* regulatory mutant strains, expression of *sarZ* is activated only by *mgrA*. Inactivation of *sarZ* does not have a major effect on the transcription of most of the other members of the *sarA* family of genes, except for *sarS*. Analysis of various double mutants of *sarZ* with other regulatory genes suggests that *sarZ* has discernible effects on the expression of *agr* RNAII and RNAIII. As *sarA* family genes are involved in the regulation of virulence genes, the expression of various potential target genes (*spa*, *hla*, and *sspA*) in *sarZ*, *sarX*, *mgrA*, *sarZ* plus *mgrA*, and *sarX* plus *sarZ* mutants were evaluated. The results show that *sarZ* is involved in the regulation of several virulence genes, particularly those genes encoding exoproteins (proteases and hemolysins).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. Phage ϕ11 was used as a generalized transducing phage for *S. aureus* strains. *S. aureus* strain RN4220, a restriction-deficient derivative of strain 8325-4, was used as the initial recipient for the transformation of plasmid constructs. *S. aureus* strains were routinely cultured in tryptic soy broth or tryptic soy agar medium supplemented with appropriate antibiotics when necessary. Luria-Bertani medium was used for growing *Escherichia coli*. For verification and routine maintenance, various *S. aureus* strains were grown with appropriate antibiotic selection as follows: 5 μg/ml of erythromycin, 50 μg/ml of kanamycin, 5 μg/ml of tetracycline, and 10 μg/ml of chloramphenicol. For *E. coli*, ampicillin at 50 μg/ml, chloramphenicol at 30 μg/ml, erythromycin at 200 μg/ml, and spectinomycin at 75 μg/ml were used.

Genetic manipulations in *E. coli* and *S. aureus*. Using a genome scan for homology with SarA, the *sarZ* gene product (SA2174) was initially identified in the *S. aureus* N315 genome database (24). To construct a *sarZ* mutant, the *sarZ* gene, together with 865-bp and 920-bp flanking sequences upstream and downstream, respectively, was amplified by PCR with the primers 5′-AACGGATCA AGTCATTTAGCA-3′ and 5′-TCTGACATCACTCAATTATATCAGA-3′, using chromosomal DNA from strain RN6390 as the template. The 2,235-bp PCR fragment was cloned into cloning vector pCR 2.1 (Invitrogen, San Diego, CA) in *E. coli*. The BamHI/XhoI DNA fragment-containing 2.3 kb fragment was then cloned into the BamHI and SalI sites of pUC19 (pAM927). A 191-bp fragment, comprising the promoter and N-terminal regions of the *sarZ* coding region, was deleted by restriction with AflII and SwaI and then replacement of the deleted fragment with an ~1.2-kb *ermC* gene at these sites (pAM945). The 3.3-kb fragment containing the *erm*C insertion fragment within the deleted *sarZ* region was cloned into the temperature-sensitive shuttle vector pCL52.2 (pAM950) (26). Construction and selection of the putative *sarZ* mutant (tetracycline-sensitive and erythromycin-resistant colonies) was performed as described previously (30, 32–34). A phage ϕ11 lysate of the putative *sarZ* mutant was then prepared to infect fresh RN6390 and SH1000 or other clinical strains to reconstruct the *sarZ* mutant in an attempt to avoid any putative genomic mutations that might have occurred during the temperature shift to promote homologous recombination. To construct double mutants of *sarZ* with *mgrA*::*ermC* and *sarX*::*ermC*, we cloned deletion (open reading frame [ORF]) and insertion (kanamycin gene cassette) fragments (e.g., *sarZ*::*kan*) into the temperature-sensitive vector pCL52.2 (pAM1055). Construction of double mutants was performed using essentially the same protocol described for single-mutant construction, except the recipient hosts were *sarX* and *mgrA* mutants. The correct mutation was confirmed by PCR and Northern and Southern hybridization with a *sarZ* probe as described previously (30, 32–34).

To complement the *sarZ* mutation, a 1.6-kb fragment encompassing the *sarZ* gene and 280 bp upstream of the *sarZ* translation start site was amplified with PCR (using primers 5′-AACGGATCAAGTCATTTAGCA-3′ and 5′-AGAAA TCGAATTACATACACGATGC-3′) and cloned into the shuttle plasmid pSK236 (pAM975) (16) or a single-copy integration vector, pCL84 (pAM980) (25). Single-copy integration into the RN6390 *sarZ* mutant was performed as described previously (33, 34). The recombinant pSK236 derivative plasmid (pAM975) was electroporated into RN4220 and selected for on chloramphenicol plates. The plasmid from RN4220 was then electroporated into the parental strain RN6390 and the *sarZ* mutant to construct *trans*-complemented strains.

Isolation of RNA and Northern blot hybridization. Total RNA from *S. aureus* was prepared by using a Trizol isolation kit (Gibco-BRL, Gaithersburg, MD) and a reciprocating shaker, as described previously (26–31). The optical densities at 600 nm (OD_{600}s) of various cultures were measured in a spectrophotometer (Spectronic 20). The concentration of total RNA was determined by measuring the absorbance at 260 nm. Ten micrograms of total RNA was analyzed by Northern blotting, as described previously (30, 32–36). The genes *agr* RNAII, *agr* RNAIII, *sarA* paralogs, *spa*, *hla*, *sspB*, and *sspA* were either amplified by PCR or excised with restriction endonucleases from the plasmids containing the desired genes. For detection of specific transcripts, gel-purified DNA probes were radiolabeled with [α-^{32}P]dCTP by using the random-primed DNA-labeling kit (Roche Diagnostics GmbH) and hybridized under aqueous-phase conditions at 65°C. The blots were subsequently washed, exposed to a phosphorimager screen,

TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Description	Source or reference
RN4220	Restriction-deficient transformation recipient	40
RN6390	Wild-type laboratory strain related to 8325-4, prophage-cured strain from human isolate NCTC8325 harboring an 11-bp deletion in *rsbU* that regulates *sigB* activity by activating RsbV, a factor that competitively binds to the anti-sigma factor RsbW	40
ALC488	*sarA* mutant of RN6390 with *sarA*::*ermC*	31
ALC1713	*sarR* mutant of RN6390 with *sarR*::*ermC*	30
ALC1927	*sarS* mutant of RN6390 with sarS::ermC	9
ALC1905	*sarT* mutant of RN6390 with *sarT*::*ermC*	49
ALC2272	*sarU* mutant of RN6390 with *sarU*::*ermC*	32
ALC2319	*sarV* mutant of RN6390 with *saV*::*ermC*	33
AM 1080	*sarX* mutant of RN6390 with *sarX*::*ermC*	34
AM 1090	*sarZ* mutant of RN6390 with *sarZ*::*ermC*	This study
ALC2530	*mgrA* mutant of RN6390 with *mgrA*::*ermC*	33
AM 1102	*rot* mutant of RN6390 with *rot*::*ermC*	This study
RN6911	*agr* mutant of RN6390 with *agr*::*tetM*	40
AM1126	*sarZ agr* double mutant of RN6390 with *sarZ*::*kan* and *agr*::*tetM*	This study
AM1175	*sarZ mgrA* double mutant of RN6390 with *sarZ*::*kan* and *mgrA*::*ermC*	This study
AM1176	*sarZ sarX* double mutant of RN6390 with *sarZ*::*kan* and *sarX*::*ermC*	This study
AM1194	AM1090 complemented with 1.6-kb *sarZ* fragment into the *geh* locus on the chromosome	This study
AM1390	AM1090 with pSK236 containing 1.6-kb *sarZ* gene at BamHI and XhoI sites	This study
SH1000	Functional *rsbU* derivative of 8325-4 *rsbU*$^+$	19
AM1397	*sarZ* mutant of SH1000 *sarZ*::*ermC*	This study
AM1398	AM1397 complemented with the *sarZ* gene into the *geh* locus on the chromosome	This study
AM1410	*mgrA* mutant of SH1000 *mgrA*::*ermC*	This study
AM1412	AM1410 with pSK236 containing 1.5-kb *mgrA* fragment at EcoRI site	This study
Newman	A human clinical isolate	37
AM1419	*mgrA* mutant of Newman *mgrA*::*ermC*	This study
AM1422	AM1419 with pSK236 containing 1.5-kb *mgrA* gene at EcoRI site	This study
Plasmids		
pUC18	*E. coli* cloning vector	
pCR2.1	*E. coli* cloning vector for direct cloning of PCR product	Invitrogen
pCL52.2	Temperature-sensitive *E. coli-S. aureus* shuttle vector	26
pCL84	Single-copy integration *E. coli-S. aureus* shuttle vector	25
pSK236	*E. coli-S. aureus* shuttle vector	16
pAM927	pUC19 containing a 2.3-kb BamHI- XhoI fragment containing *sarZ* region at BamHI-SalI sites	This study
pAM945	pUC19 containing ~3.3-kbDNA fragment that has a deletion of a 191-bp AflII/SwaI fragment that includes promoter and N-terminal (31-residue) regions of the *sarZ* gene and an insertion of the 1.2-kb *ermC* gene	This study
pAM950	pCL52.2 containing ~3.3-kbDNA fragment *sarZ::ermC* from pAM945	This study
pAM1055	Same as pAM945, but the *ermC* gene was replaced with 1.2-kb kanamycin gene (*kan*)	This study
pAM975	pSK236 with a 1.6-kb DNA fragment containing the *sarZ* gene with 280-bp upstream sequence	This study
pAM980	pCL84 with a 1.6-kb DNA fragment containing the *sarZ* gene with 280-bp upstream sequence	This study
pAM1110	pSK236 with a 1.5-kb DNA fragment containing the *mgrA* gene region	

and scanned on a phosphorimager. The intensities of the bands were quantified with Image Quant software (Amersham Life Sciences).

Purification of SarZ and MgrA proteins and production of anti-SarZ polyclonal antibodies. The cloning and purification of a six-His tag–MgrA fusion protein was described previously (33). The 450-bp DNA fragment containing the full-length *sarZ* gene was amplified by PCR using chromosomal DNA from *S. aureus* RN6390 as the template and primers containing flanking NdeI and BamHI restriction sites (5′-ATCATATGTATGTAGAAAACAGCTAT-3′ and 5′-ATGGATCCATACTTCTGCCCATCACCTTAT-3′) to facilitate in-frame cloning into the expression vector pET14b (Novagen, Madison, WI). The recombinant plasmid containing the full-length *sarZ* coding region was confirmed by restriction digestion and DNA sequencing and transformed into *E. coli* BL21(DE3)/pLysS. His_6-SarZ protein expression and purification were done in a manner similar to those for the SarA protein, as described previously (12). The purity of the His-SarZ fusion protein was checked in a sodium dodecyl sulfate (SDS)-12% polyacrylamide gel and found to be more than 98% pure. The concentration of the purified protein was determined by using a Bio-Rad (Hercules, CA) protein estimation kit with bovine serum albumin as the standard.

Anti-SarZ sera were prepared by immunizing two (BALB/c × SJL/J)F_1 mice with 100 μg per mouse of purified His-SarZ fusion protein as described previously (30). Western blotting was performed to monitor the titers of the immune sera.

Gel shift analysis. A 270-bp DNA fragment encompassing the *sarZ* promoter region upstream of the translational start site (ATG) was PCR amplified by using chromosomal DNA from RN6390 with the primers 5′-AGAAATCGAATTAC ATACACGATGC-3′ and 5′-ACATCCAATCACTCCTTGTTAA-3′ and cloned into the pCR2.1 vector (Invitrogen, CA). The DNA fragment was excised by digestion, gel purified, and dephosphorylated. In order to determine if the recombinant MgrA protein interacted with the 270-bp *sarZ* promoter fragment or if SarZ interacted with the *sarS* (9) and *sspA* (33) promoter regions, gel shift assays were performed as described previously (12, 30, 32–36). Briefly, DNA fragments were end labeled with [γ^{32}P]ATP using polynucleotide kinase. The labeled fragment (1 ng or 6 fmol) was incubated at room temperature for 20 min with various amounts of purified MgrA or SarZ protein in 25 μl of binding buffer (25 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, 75 mM NaCl, 1 mM dithiothreitol, and 10% glycerol) containing 0.5 μg of calf thymus DNA (Amersham Pharmacia Biotech). The reaction mixtures were analyzed in an 8.0% nondenaturing polyacrylamide gel. The band shifts were detected by exposing the dried gels to X-ray films or a phosphorimager screen. The binding affinity of MgrA or SarZ protein to its cognate promoters was derived from the dissociation constant, which is

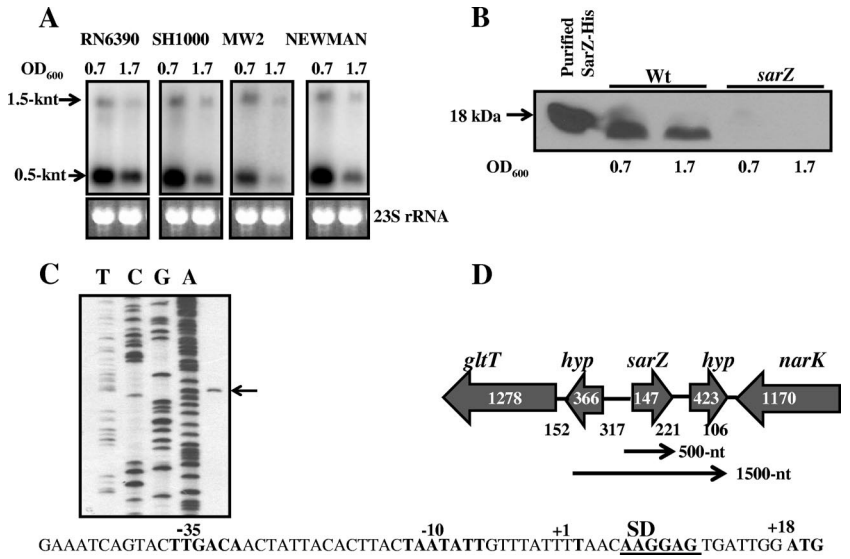

FIG. 2. Transcription, expression, and promoter analysis of the *sarZ* gene in *S. aureus*. (A) Northern analysis of the *sarZ* transcripts in the different wild-type strains at various phases of growth (an OD_{600} of 0.7 is approximately the early exponential phase of growth, and an OD_{600} of 1.7 is approximately the postexponential phase of growth). The blots were probed with 500-bp *sarZ* DNA fragments containing the entire ORF of the *sarZ* gene. The region of 23S rRNA of the ethidium bromide-stained gel used for blotting is also shown as a loading control. (B) Cell extracts of the RN6390 strain were immunoblotted onto nitrocellulose and probed with anti-SarZ polyclonal antibodies. A purified His tag fusion of SarZ was loaded as a positive control. Wt, wild type. (C) Primer extension of the *sarZ* transcript with total RNA isolated from the wild-type RN6390 at the exponential phase of growth. The nucleotide sequence with the predicted promoter region of the *sarZ* ribosome-binding site (SD) and the translational start codon (ATG) are indicated. (D) Location of the *sarZ* gene (SA2174) on the *S. aureus* chromosome. The *sarZ* gene and the other ORFs in its vicinity are depicted. The number within each ORF indicates the size of the gene (in bp), and the number below the junction of two neighboring genes is the intergenic distance (in bp) between them. *hyp*, hypothetical ORF with unknown function. The location of a 500-nt transcript is mapped based on primer extension results, whereas the origin of the 1,500-nt transcript is hypothetical.

defined as the amount of protein needed to shift 50% of the labeled probe (K_d [dissociation constant] = $[\text{protein}]_{50\% \text{ of DNA probe}}$) (47).

Primer extension analysis. Mapping of the 5′ end of the *sarZ* transcript by primer extension was performed by using the primer 5′-CATATTCCTTAAGA TAGTTTGTG-3′, complementary to the *sarZ* coding strand and located at nucleotide positions 103 to 80 downstream from the putative proposed start codon, ATG. Primer extension was carried out by using 30 μg of total RNA isolated from the wild-type strain RN6390 in the exponential phase, as described previously (1, 30, 36). The same radiolabeled primer was used for the DNA-sequencing reactions.

Cell extract preparation and Western analysis. Cell extracts from early exponential (OD = 0.7) and postexponential (OD = 1.7) phases of growth were prepared from RN6390 and isogenic *sarZ* mutant staphylococcal strains. Cells were grown in 20 ml of tryptic soy broth medium without any antibiotics. After being pelleted, the cells were resuspended in 0.5 ml of cell lysis buffer (25 mM Tris-Cl, 5 mM EDTA [pH 8.0], 100 mM NaCl) and treated with lysostaphin. The lysostaphin-treated cells were briefly sonicated and centrifuged at 40,000 rpm for 30 min at 4°C to remove cell debris. The concentration of total proteins from clear lysates was determined by using the Bradford method (Bio-Rad, Hercules, CA).

Equal amounts of total cellular proteins were separated in an SDS-12% polyacrylamide gel and transferred onto nitrocellulose membranes as described previously (30). The blot was incubated at room temperature with a 1:1,000 dilution of anti-SarZ polyclonal antibodies for 2 h, followed by another hour of incubation with a 1:10,000 dilution of goat anti-mouse–horseradish peroxidase conjugate (Pierce, IL). Immunoreactive bands were detected as described previously with an enhanced-chemiluminescence detection kit (Piece, IL). SeaBlue prestained protein standards (Invitrogen, San Diego, CA) were used for molecular weight estimations.

Zymographic analysis. For the detection of extracellular protease activity, SDS-polyacrylamide gel electrophoresis-based zymographic analysis was performed as described by Rice et al. (46). The gels were subsequently photographed, and the white bands in the gels (zones of hydrolysis) indicated regions of protease activity.

RESULTS

Characterization of the *sarZ* gene. Analysis of *sarZ* transcription during different phases of growth was performed using Northern hybridization with total RNA isolated from various *S. aureus* strains, including laboratory-adapted strains (RN6390 and SH1000) and clinical isolates (Newman and MW2). A major *sarZ* transcript of approximately 500 nt was observed during the early exponential phase of growth (OD, ~0.7), and there was significantly reduced transcription during the postexponential phase of growth. A minor transcript of 1,500 nt was also observed, which probably originated from a cryptic promoter (Fig. 2A). We speculated that the larger transcript may play a minor role in production of SarZ or in the regulation process; therefore, further characterization of the largest transcript was not pursued. The result indicates that *sarZ* transcription is growth phase dependent (Fig. 2A). The growth phase-dependent differential transcription patterns were similar in all four tested strains, including RN6390, a strain that produces less SigB due to a natural 11-bp deletion in a putative *sigB* activator, *rsbU*. The SarZ protein per se had not been shown to be expressed in *S. aureus*. To detect the SarZ protein in *S. aureus* and to investigate whether the transcription pattern correlated with the expression of the protein, Western blot analysis with the cell extracts of wild-type RN6390 and an isogenic *sarZ* mutant was performed (Fig. 2B). The results showed reduced expression of SarZ (17.5 kDa) in the postexponential phase of growth compared to the early

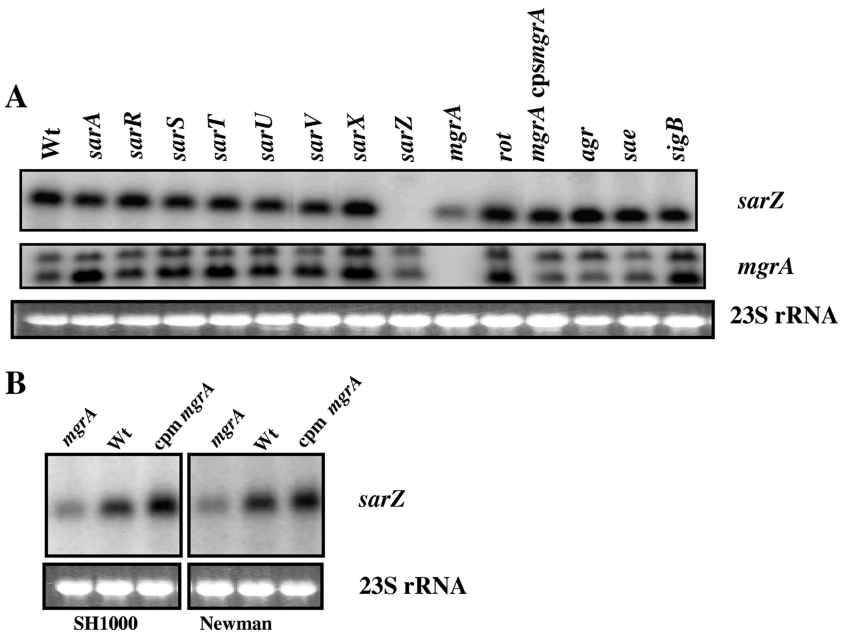

FIG. 3. (A) Northern analysis of the *sarZ* and *mgrA* transcripts in the wild type (Wt), various isogenic mutants, and a single-copy complemented strain of the *mgrA* mutant at exponential phase (OD_{600}, ~0.7) of growth. DNA fragments (500 bp and 550 bp) containing *sarZ* and *mgrA* genes, respectively, were used for hybridization. cps*mgrA* indicates the complementation in single copy of the *mgrA* gene on the lipase locus (*geh*) of the *mgrA* mutant. (B) Northern analysis with a 500-bp *sarZ* DNA probe of the *mgrA* mutants and complemented (cpm) strains from different *S. aureus* strains, as indicated. The region of 23S rRNA of the ethidium bromide-stained gel used for blotting is also shown as a loading control in both panels.

exponential phase of growth. As expected, the extracts from the *sarZ* mutant did not react with the SarZ antiserum. These results clearly show that SarZ expression is growth phase dependent and that maximal expression occurs in the early exponential phase of growth.

In order to determine the transcriptional start site and the promoter structure of the major *sarZ* transcript, primer extension analysis was performed with total RNA isolated from the wild-type RN6390 (Fig. 2C). The transcription start site was mapped to a "T" located 17 nt upstream from the predicted SarZ ATG initiation codon in *S. aureus* (Fig. 2C). The predicted promoter regions and the ribosome binding site are also depicted in Fig. 2C. Based upon the transcriptional start site, the predicted promoter regions closely resemble the −35 and −10 consensus sequences of σ^A-dependent promoters. The physical location of the *sarZ* ORF on the staphylococcus genome is shown in Fig. 2D. The *sarZ* ORF is flanked by a hypothetical ORF on each side, while *gltT* (encoding a proton/sodium-glutamate symport protein; SA2172) and *narK* (encoding a nitrite transporter; SA2176) are further upstream and downstream, respectively. SarZ is highly conserved among the sequenced strains of *Staphylococcus* (97 to 100% identity among 11 strains of *S. aureus*). Orthologues of SarZ are also present in the genomes of two *S. epidermidis* strains (79% identity), one *Staphylococcus saprophyticus* strain (70% identity), and one *Staphylococcus haemolyticus* (65% identity) strain.

Regulatory relationship between different *sarA* family genes. Previous characterization of *sarA* family genes has shown that most of these genes are regulated by other members of their own family. For example, *sarV* transcription, which is undetectable or very low under normal growth conditions in the wild-type strain, is significantly increased in a *sarA* or *mgrA* mutant (33). To determine the influence of *sarZ* on other members of the *sarA* family and to elucidate the phenotypic effects of *sarZ* inactivation, a *sarZ* mutant was constructed by allelic replacement as described in Materials and Methods. Northern blot assays were performed with a *sarZ* probe (500 bp, encompassing the coding region) with various *sarA* family mutants to determine if the transcription of *sarZ* was affected by any of the other *sarA* family genes. The Northern blotting results disclosed that the level of *sarZ* transcription was reduced threefold in the *mgrA* mutant, whereas transcription was not affected in the other nine *sarA* family mutants tested (Fig. 3A). Upon introduction of the functional *mgrA* gene into the *mgrA* mutant, the expression of the *sarZ* transcript returned almost to the parental level (Fig. 3A), indicating that the transcription of *sarZ* was activated by the *mgrA* gene product. To test if the above-described mode of regulation was also present in other *S. aureus* backgrounds, *mgrA* mutants were constructed in strains SH1000 and Newman. The mutants were also complemented with functional copies of the *mgrA* gene, and the expression of *sarZ* was assessed. The results showed a reduction in *sarZ* transcripts in both SH1000 and Newman *mgrA* mutants while near-normal levels of expression were observed in the complemented mutants (Fig. 3B). Therefore, the results of these analyses suggested that the expression of *sarZ* was activated by *mgrA* in all tested backgrounds in *S. aureus*.

Binding of MgrA to the *sarZ* promoter region. The direct interaction of MgrA with the *sarZ* promoter was tested in gel shift assays using purified MgrA protein and a 270-bp DNA fragment containing the *sarZ* promoter region (Fig. 4). Re-

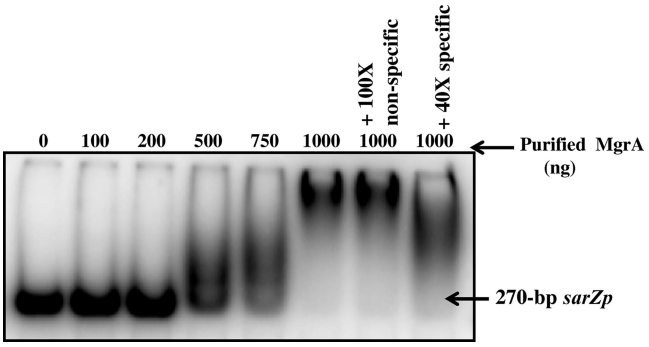

FIG. 4. Autoradiogram of an 8.0% polyacrylamide gel showing gel shifts for purified MgrA protein with a γ-^{32}P-labeled 270-bp *sarZ* promoter fragment (1 ng or 6 fmol per lane). The mobility of the band was noted in the presence of increasing amounts of MgrA protein, as indicated above the gel. The lanes containing competitor DNA, i.e., an unlabeled specific 270-bp *sarZ* promoter fragment (40-fold molar excess) and a nonspecific 185-bp internal DNA fragment of the *sarX* ORF (100-fold molar excess) are indicated.

tarded protein-DNA complex could be detected with 0.5 μg of MgrA (K_d, ~13 nM, assuming that MgrA is a dimeric protein) (47), and complete conversion occurred with 1.0 μg of MgrA. The unlabeled promoter fragment could compete with the labeled fragment, whereas a nonspecific, unrelated DNA fragment (a 185-bp fragment from the internal region of the *sarX* gene) did not compete with the labeled fragment (Fig. 4), indicating that DNA-protein interaction was specific. Previously, the DNA binding motif (GTTG) of the MgrA protein had been mapped on the *sarV* promoter region by DNase I footprinting (33). DNA sequence analysis upstream of the *sarZ* promoter region revealed the presence of the sequence in **ACTTGACAAC**T, which is consistent with MgrA binding motifs (shown in bold) on promoter regions of other *mgrA*-regulated genes (33, 34). Interestingly, the putative MgrA binding site overlaps with the −35 promoter region of the *sarZ* gene (Fig. 2C). Hence, the MgrA protein can bind to the *sarZ* promoter region, presumably acting as an activator of *sarZ* transcription.

Transcription of regulatory genes in a *sarZ* mutant. To determine whether inactivation of *sarZ* affects other regulatory genes, particularly the members of the *sarA* family and *agr*, Northern blot analysis with probes for nine *sarA* paralog genes (*sarA*, *sarR*, *sarS*, *sarT*, *sarU*, *sarV*, *sarX*, *mgrA*, and *rot*), *agr* RNAII, and *agr* RNAIII was performed. Among the *sarA* family genes monitored, only transcription of *sarS* was considerably elevated (fourfold compared to the wild type) due to inactivation of the *sarZ* gene (Fig. 5A). Several members of the *sarA*, family such as *sarA* and *mgrA*, showed minor variation (~20% to 30%) in transcription (data not shown), which is consistent with the slightly increased SarA expression observed in a recent study (51). Upon single-copy complementation of the wild-type *sarZ* gene to a *sarZ* mutant, the level of *sarS* transcription was restored to nearly the parental level; con-

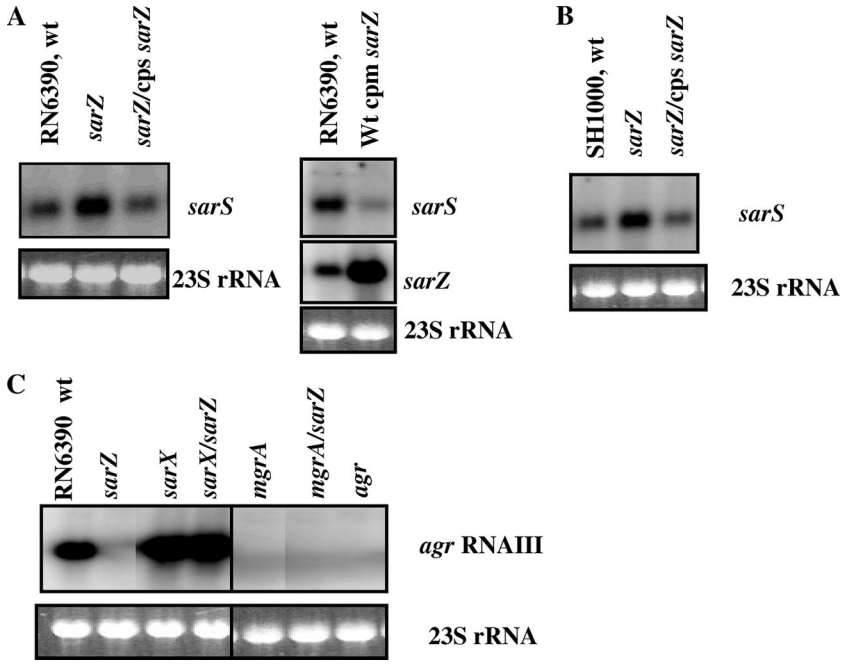

FIG. 5. Analysis of the expression of *sarS* and *agr* transcripts in various mutant strains. (A) Northern blots of *sarS* transcript in the wild-type (wt), *sarZ* mutant, and single-copy (cps) complemented strains from the mid-exponential (OD_{600}, ~1.1) phase of growth in an RN6390 background. (Right) Blots of *sarS* and *sarZ* transcripts in the wild type and the wild type expressing the *sarZ* gene in a multicopy shuttle vector, pSK236 (cpm), from the mid-exponential (OD_{600}, ~1.1) phase of growth. The blots were hybridized with 750-bp and 500-bp DNA fragments containing ORFs of *sarS* and *sarZ*, respectively. (B) Northern blots of *sarS* transcript in the wild-type, *sarZ* mutant, and single-copy (cps) complemented strains from the mid-exponential (OD_{600}, ~1.1) phase of growth in an SH1000 background. (C) Northern blots of *agr* RNAIII transcript in the wild type and various single and double mutants, as indicated, from the postexponential (OD_{600}, ~1.7) phase of growth. The blots were probed with 0.5-kb DNA fragments containing the *agr* RNAIII region. The region of 23S rRNA of the ethidium bromide-stained gel used for blotting is also shown as a loading control in all panels.

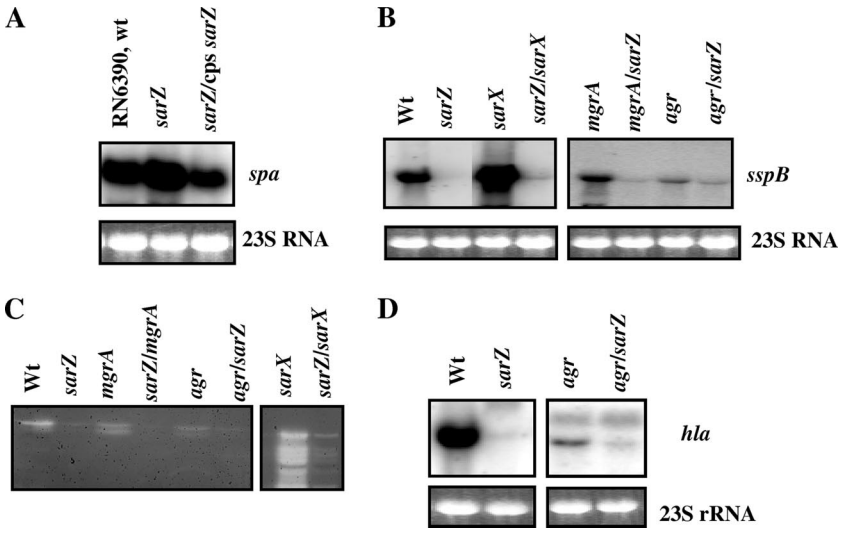

FIG. 6. Analysis of target gene transcription (A, B, and D) and expression (C) in different isogenic single- and double-mutant strains of RN6390. (A) Northern blots of *spa* (protein A) transcript in the RN6390 wild-type (wt), *sarZ* mutant, and single-copy (cps) complemented strains from the mid-exponential (OD_{600}, ~1.1) phase of growth. (B) Northern blots of cysteine protease (*sspB*) transcript in the wild-type and isogenic single- and double-mutant strains, as indicated, from the postexponential phase of growth (OD_{600}, ~1.7). A 1.0-kb DNA fragment containing the *sspB* ORF was used for hybridization. In *S. aureus*, the V8 protease gene (*sspA*), the cysteine protease gene (*sspB*), and an unknown gene (*sspC*) are in a single transcriptional unit (39). (C) Gelatin zymogram of culture supernatants from various *S. aureus* RN6390 strains as indicated. Equal amounts of culture supernatant (OD_{600}, ~1.7) were used for all strains, except *sarX* and the *sarZ sarX* mutant, where one-fifth volume was applied. (D) Northern blot analysis for α-hemolysin (*hla*) transcript in assorted *S. aureus* RN6390 strains, as indicated, from the postexponential phase of growth. The Northern blot was hybridized with an *hla* fragment containing the coding region of the hemolysin gene. The region of 23S rRNA of the ethidium bromide-stained gel used for blotting is also shown as a loading control in panels A, B, and D.

firming that *sarZ* activates *sarS* expression. To further confirm that *sarZ* was indeed involved in the regulation of *sarS*, the *sarZ* gene was overexpressed in a multicopy shuttle vector in the wild-type strain RN6390 (Fig. 5A, right). The level of *sarS* transcription was decreased (~6-fold) in the overexpressing *sarZ* strain compared to the parental strain. Hence, these results clearly indicate that the level of *sarS* transcription is directly regulated by the *sarZ* gene product. The transcription of *sarS* was monitored in the SH1000 *sarZ* mutant, which is *rsbU*$^+$ and belongs to the same lineage as RN6390. As shown in Fig. 5B, *sarS* transcription was elevated in the SH1000 *sarZ* mutant, and on complementation with a functional single-copy *sarZ* gene, *sarS* transcription was restored to nearly the parental level.

Previous reports showed that the *agr* locus was positively regulated by SarZ and MgrA (11, 20, 51), while the same locus was negatively regulated by SarX (34). To understand the interregulatory relationship between *mgrA*, *sarX*, and *sarZ* with respect to *agr* regulation, *mgrA sarZ* and *sarZ sarX* double mutants were constructed and the expression of *agr* transcripts was monitored. Northern analysis with a 0.5-kb *agr* RNAIII probe with the total RNA isolated from the wild type and various isogenic single (*sarZ*, *sarX*, and *mgrA*) and double (*sarZ mgrA* and *sarZ sarX*) mutants of the *sarZ* gene was performed (Fig. 5C). Transcription of *agr* RNAIII was severely diminished (~10-fold) in the *sarZ* mutant but enhanced (>3-fold) in the *sarX* mutant compared with the wild type. Interestingly, *agr* RNAIII expression in the *sarZ sarX* double mutant was similar to that of the *sarX* mutant, suggesting that *sarX*-mediated *agr* repression was probably independent of *sarZ*. The expression of *agr* was virtually undetectable in the *mgrA* or *mgrA sarZ* mutant (Fig. 5C). A similar regulatory pattern was observed in the case of *agr* RNAII in these mutant strains (data not shown), thus demonstrating the involvement of *sarZ*, *sarX*, and *mgrA* gene products in *agr* expression.

Transcriptional regulation of the selected target genes in a *sarZ* mutant. The data presented above clearly showed that inactivation of *sarZ* affected the expression of *sarS* and *agr* transcription in opposite ways. The expression of genes regulated by *sarS* and *agr* target genes was assessed. Transcription of *spa* (protein A), a gene activated by SarS, was enhanced in the *sarZ* mutant and on complementation; the level of *spa* transcription was reduced almost to parental levels (Fig. 6A). The expression of a few virulence genes, particularly those for exoproteins, known to be regulated by *agr*-dependent or -independent mechanisms was also monitored in the *sarZ* single mutant, as well as *sarZ sarX*, *sarZ mgrA*, and *sarZ agr* double-mutant backgrounds. Among the genes tested were those encoding proteases (a V8 protease [*sspA*] and a cysteine protease [*sspB*]) and hemolysin (e.g., *hla*). The protease (*sspB*) expression (*sspA* and *sspB* are part of the same operon) (39) was severely reduced in *sarZ* (~9-fold), *mgrA* (3-fold), and *agr* (6-fold) mutants (Fig. 6B). Deletion of *sarZ* in an *mgrA* or *agr* mutant background led to further 9- and 3.5-fold reductions, respectively, in the expression of *sspB* transcript compared to the corresponding single mutant. Expression of *sspB* was enhanced 3-fold in the *sarX* mutant, while a significant reduction (~27-fold) of the same transcript was observed in the *sarZ sarX* double mutant, suggesting that protease regulation is dependent on *sarZ*. Phenotypic characterization in a gelatin-containing gel also showed that the *sarZ* mutant produced lower levels of proteases than the wild type. In all the double

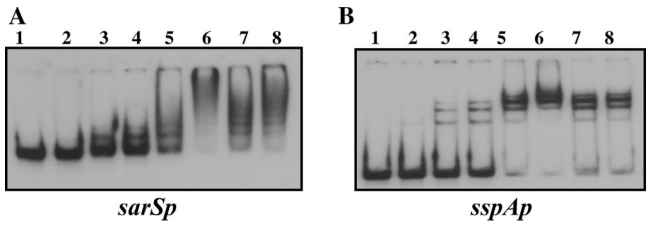

FIG. 7. DNA binding activity of SarZ protein. Autoradiograms of 8.0% polyacrylamide gels showing the binding of SarZ protein to 287-bp and 250-bp promoter fragments (1 ng each or 5 and 6 fM, respectively) of *sarS* (A) and *sspA* (B), respectively. In panel A, lanes 1 to 5 correspond to 0 ng, 100 ng, 200 ng, 300 ng, and 500 ng, and lanes 6 to 8 correspond to 1.0 μg of purified SarZ protein. In panel B, lanes 1 to 5 correspond to 0 ng, 25 ng, 50 ng, 100 ng, and 200 ng, and lanes 6 to 8 correspond to 300 ng of purified SarZ protein. The mobilities of the bands were noted in the presence of increasing amounts of SarZ protein. A 50-fold excess (molar ratio) of specific unlabeled competitor DNA of the respective promoter fragments was used for competition in lanes 7 in both panels, whereas a 50-fold molar excess of a nonspecific competitor DNA (a 185-bp internal fragment of the *sarX* ORF region) is shown in lane 8 of each panel.

mutants, the expression of protease was lower than that in the single mutant alone (Fig. 6C). The *sarX* mutant showed very high protease activity, but protease expression in the *sarZ sarX* double mutant was considerably lower than in the *sarX* single mutant, which was in good agreement with results obtained from transcriptional analysis. Similarly, a reduction of 15-fold in the transcription of α-hemolysin (*hla*) in the *sarZ* mutant with respect to the wild type was observed. The *sarZ agr* double mutant showed a threefold reduction in *hla* transcription compared to the *agr* mutant alone (Fig. 6D), suggesting the presence of an *agr*-independent mode of *hla* regulation by SarZ.

Binding of SarZ protein to the promoter regions of various *sarZ*-regulated target genes. Since *sarS* and *sspB* transcription is altered in the *sarZ* mutant, the ability of SarZ to bind directly to the *sarS* or *sspAB* promoter regions was tested. The purified SarZ protein was used for the gel shift assays with a 287-bp *sarSp*- and a 250-bp *sspAp*-labeled DNA fragment containing the respective promoter regions (Fig. 7). Retarded DNA-protein complex was detected with as little as 200 ng or 50 ng of SarZ protein for *sarSp* and *sspAp* promoter fragments, respectively. As the concentration of SarZ increased, the retarded protein-DNA complexes became the predominant band, with complete conversion at 1.0 μg and 0.3 μg of SarZ protein for the *sarS* and *sspA* promoter fragments, respectively. Unlabeled specific competitor DNA fragments, as well as nonspecific competitor DNA, could eliminate retarded complexes equally for *sarSp* and *sspAp* promoter fragments. This indicates that SarZ has a nonspecific DNA binding activity, which is also consistent the observation made earlier by Kaito et al. (20) in gel shift assays with *agr* and *hla* promoter regions and the purified SarZ protein.

DISCUSSION

All living organisms process various signals through complex regulatory pathways. Thus, insight into how the behavior of a bacterial cell is controlled by its regulatory networks requires a detailed understanding of the individual regulatory components. *S. aureus* exhibits complex patterns of protein expression in response to various diverse environmental conditions, which are coordinately regulated by large numbers of regulatory networks (11, 18, 41). One such regulatory pathway that controls the expression of genes involved in pathogenesis, metabolic processes, antibiotic resistance, and biofilm formation involves the SarA protein family (consisting of 10 paralogous proteins) in *Staphylococcus* (11). In this report, we have examined the expression, regulation, and function of the *sarZ* gene.

The expression of the *sarA* paralogs is variable and in many cases depends on the phase of growth. For example, under normal laboratory growth in broth medium, *sarA* and *mgrA* transcripts are highly expressed, whereas the transcripts of *sarU*, *sarT*, and *sarV* are not readily detectable and *sarR*, *rot*, *sarS*, and *sarX* are expressed moderately in a growth phase-dependent manner. Although the preliminary characterization of the *sarZ* gene has been described previously (20, 44, 51, 56), the regulation of *sarZ*, especially its relationship with other members of the *sar* family and the characterization of *sarZ* transcription (e.g., transcriptional and translational start sites), were not well defined. Moreover, the actual production of SarZ protein had not been demonstrated in *S. aureus*. To address these issues, transcriptional analysis of *sarZ* transcript was performed and SarZ-specific antiserum was raised to detect the *sarZ* gene product in *S. aureus*. *sarZ* transcription was observed to be maximal in the early exponential phase of growth but declined in the postexponential phase. Employing the SarZ antiserum, the SarZ protein was clearly detected in whole-cell extracts of RN6390, and as with *sarZ* transcripts, more SarZ production was observed in the exponential phase than in the postexponential phase (Fig. 2). Indeed, expression analysis of the *sarZ* gene in various laboratory isolates, as well as four clinical isolates (Newman, MW2, COL, and UAMS-1), suggested a similar pattern of *sarZ* expression (unpublished data).

Based on the primer extension experiment, the predicted promoter was found to be σ^A dependent, which agrees with the observation that most of the major *sar*-family gene transcripts are expressed by the σ^A promoter (11, 30, 32–36, 52). Analysis of the sequence downstream of the *sarZ* gene disclosed the presence of a potential hairpin structure with an 8-bp inverted-repeat sequence, which is a *rho*-independent transcriptional terminator sequence. Thus, the Northern and Western studies suggest that SarZ is composed of 148 residues, as predicted in various annotations of *Staphylococcus* genomes. The SarZ protein is present in all of the sequenced *Staphylococcus* genomes and is more similar to MgrA (34% identity and 63% homology) than to SarA (28% identity and 53% homology). Among SarA family proteins, MgrA and SarZ are more similar to the MarR family of proteins among gram-negative organisms (e.g., 19% identity with *E. coli* MarR, 34% identity with *Bacillus subtilis* OhrR, and 21% identity with *Pseudomonas aeruginosa* MexR) and consist of six α-helices and two β-sheets.

It is well established that members of the SarA family regulate other members of their own family, as well as other regulatory systems and many target genes in *S. aureus*. Among 13 regulatory mutants (10 *sar* family mutants, *agr*, *sae*, and *sigB*) tested, a decrease in expression of *sarZ* transcript was observed only in an *mgrA* mutant, and transcription was restored to the parental level on complementation with the *mgrA* gene (Fig. 3A). The pattern of *mgrA*-mediated regulation was

similar in another pair of laboratory (SH1000) and clinical (Newman) isolates, both of which produce more of the stress-responsive SigB factor. In gel shift studies, MgrA could bind to the *sarZ* promoter region. Analysis of the consensus MgrA binding motif (GTTG) suggested the presence of two similar binding motifs within the −35 region of the *sarZ* promoter, and it is possible that MgrA aids the binding of RNA polymerase to the promoter to enhance *sarZ* gene expression. Inactivation of *sarZ* did not have any major effect on the transcription of other *sarA* family genes, except for activation of *sarS* transcription. In fact, overexpression of *sarZ* in a wild-type strain led to a drastic (~6-fold) decrease in expression of *sarS* transcription. These results suggest that SarZ is a repressor of *sarS* transcription in *S. aureus*, which was supported by the DNA binding ability of SarZ to the *sarS* promoter. However, these results should be interpreted with caution, as the ability of SarZ to bind the *sarS* promoter was equally inhibited by specific and nonspecific competitor DNA (Fig. 7). This suggests that in vitro SarZ has a nonspecific DNA binding activity, which is also consistent with the observation made by Kaito et al. (20). SarS is known to activate the expression of *spa* (protein A), and the *sarZ* mutant that has elevated *sarS* transcription showed enhanced transcription of *spa*. This indicates that SarZ regulates *spa* expression, probably via SarS, which is consistent with the inability to identify SarZ protein during an affinity pull-down assay with the *spa* promoter fragment and cell lysate of strain 8325-4 (42).

The results from an earlier study showed that SarX is the only negative regulator of *agr* (34), while MgrA (12) and SarZ are positive regulators of *agr* transcription. In this study, too, inactivation of *sarZ* drastically reduced *agr* expression, while *sarX* deletion enhanced *agr* expression. In the *sarZ sarX* double mutant, *agr* expression was virtually indistinguishable from that of the *sarX* single mutant. These results clearly suggest the involvement of *sarX*, *mgrA*, and *sarZ* gene products in *agr* regulation. It is possible that SarZ could be required to counter the repressive effects of SarX. Thus, if *sarX* is absent, then *sarZ* is not required for *agr* expression and enhanced *agr* expression is observed due to the absence of *sarX*-mediated repression (Fig. 5C). This raises the interesting possibility that SarZ and SarX may regulate *agr* by interacting with each other. Preliminary data employing in vitro protein-protein interaction assays show that SarZ can directly interact with SarX (unpublished data).

The *ssp* locus is important for virulence in *S. aureus*, and proteases have been implicated in spreading of *S. aureus* in host tissues (46). In addition, the proteases have been shown to contribute to in vivo growth and survival of *S. aureus* in different infection models. Hence, the study of *ssp* regulation is of particular importance in *S. aureus* pathogenesis. In *S. aureus*, *agr* RNAIII activates *ssp* expression, while several of the *sar* family genes repress its expression. The results presented here (Fig. 6B) clearly show that *sarZ* mutants have reduced *agr*, as well as *sspB*, expression. If *sarZ* regulates *sspB* expression only via *agr*, then *sspB* expression in *agr* single mutants or *sarZ agr* double mutants should remain the same. Nevertheless, there is a reduction in *sspB* expression (~3.5-fold) in the double mutant compared to the single mutant. Moreover, both the *sarX* single mutant and the *sarZ sarX* double mutant produce more *agr* transcripts than the wild type or the *sarZ* mutant. However, in spite of high *agr* expression, the expression of *sspB* is reduced in the *sarZ sarX* double mutant. This clearly implies that *sspB* is regulated by *sarZ* independently of *agr*.

To summarize briefly, we have characterized the expression of the *sarZ* gene and have shown that MgrA is required for optimal *sarZ* expression. We have also shown that *sarZ* upregulates *spa* transcription by enhancing *sarS* expression. This suggests that the *sar* family genes are regulated by other members of their own family (Fig. 1). Analysis of a *sarZ* knockout in combination with other regulatory genes (e.g., *agr* and *sarX*) suggests that *sarZ* can regulate the expression of virulence genes, in particular, *sspB* and *hla* genes, in an *agr*-independent manner. These results are consistent with the recent finding that *sarZ* inactivation leads to increased transcription of *spa* and downregulation of *hla* and *sspA* transcripts in the RN6390 strain (51). All these results demonstrate the central regulatory role played by *sarZ* in controlling the regulatory and virulence genes in *S. aureus*. One useful way to target the bacterial cells in vivo may be to disrupt the regulatory systems, thereby minimizing the expression of cell wall-associated and extracellular proteins. Therefore, by blocking the functions of critical regulators, such as SarZ, one can expect to minimize the expression of genes involved in virulence and survival.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 2000. Current protocols in molecular biology. John Wiley & Sons, New York, NY.
2. **Blevins, J. S., A. F. Gillaspy, T. M. Rechtin, B. K. Hurburt, and M. S. Smeltzer.** 1999. The staphylococcal accessory regulator (*sar*) represses transcription of the *S. aureus* collagen adhesin gene (*cna*) in an *agr*-independent manner. Mol. Microbiol. **33:**317–326.
3. **Brandenberger, M., M. Tschierske, P. Giachino, A. Wada, and B. Berger-Bachi.** 2000. Inactivation of a novel three-cistronic operon *tcaR-tcaA-tcaB* increases teicoplanin resistance in *Staphylococcus aureus*. Biochim. Biophy. Acta **1523:**135–139.
4. **Brunskill, E. W., and K. W. Bayles.** 1996. Identification of LytSR-regulated genes from *Staphylococcus aureus*. J. Bacteriol. **178:**5810–5812.
5. **Cassat, J., P. M. Dunman, E. Murphy, S. J. Projan, K. E. Beenken, K. J. Palm, S.-J. Yang, K. C. Rice, K. W. Bayles, and M. S. Smeltzer.** 2006. Transcriptional profiling of a *Staphylococcus aureus* clinical isolate and its isogenic *agr* and *sarA* mutants reveals global differences in comparison to the laboratory strain RN6390. Microbiology **152:**3075–3090.
6. **Chen, P. R., T. Bae, W. A. Williams, E. M. Duguid, P. A. Rice, and O. Schneewind, and C. He.** 2006. An oxidative-sensing mechanism is used by the global regulator MgrA. Nat. Chem. Biol. **2:**591–595.
7. **Chen, P. R., S. Nishida, C. B. Poor, A. Cheng, T. Bae, L. Kuechenmeister, P. M. Dunman, D. Missiakas, and C. He.** 2008. A new oxidative sensing and regulation pathway mediated by the MgrA homologue SarZ in *Staphylococcus aureus.* Mol. Microbiol. doi:10.1111/j.1365-2958.2008.06518.x.
8. **Cheung, A. L., J. M. Koomey, C. A. Butler, S. J. Projan, and V. A. Fischetti.** 1992. Regulation of exoprotein expression in *Staphylococcus aureus* by a locus (*sar*) distinct from *agr*. Proc. Natl. Acad. Sci. USA **89:**6462–6466.
9. **Cheung, A. L., K. A. Schmidt, B. Bateman, and A. C. Manna.** 2001. SarS, a SarA homolog repressible by *agr*, is an activator of protein A synthesis in *Staphylococcus aureus*. Infect. Immun. **69:**2448–2455.
10. **Cheung, A. L., A. S. Bayer, G. Zhang, H. Gresham, and Y.-Q. Xiong.** 2004. Regulation of virulence determinants *in vitro* and *in vivo* in *Staphylococcus aureus*. FEMS Microbiol. Lett. **1649:**1–9.
11. **Cheung, A. L., K. A. Nishina, M.-P., Trotonda, and S. Tamber.** 2008. The SarA protein family of *Staphylococcus aureus*. Int. J. Biochem. Cell Biol. **40:**355–361.
12. **Chien, Y.-T., A. C. Manna, S. J. Projan, and A. L. Cheung.** 1999. SarA, a

global regulator of virulence determinants in *Staphylococcus aureus*, binds to a conserved motif essential for *sar*-dependent gene regulation. J. Biol. Chem. **274:**37169–37176.
13. **Dunman, P. M., E. Murphy, S. Haney, D. Palacios, G. Tucker-Kellogg, S. Wu, E. L. Brown, R. J. Zagursky, D. Shlaes, and S. J. Projan.** 2001. Transcriptional profiling-based identification of *Staphylococcus aureus* genes regulated by *agr* and/or *sarA* loci. J. Bacteriol. **183:**7341–7353.
14. **Fournier, B., and D. C. Hooper.** 2000. A new two-component regulatory system involved in adhesion, autolysis, and extracellular proteolytic activity of *Staphylococcus aureus*. J. Bacteriol. **182:**3955–3964.
15. **Fournier, B., A. Klier, and G. Rapoport.** 2001. The two-component system ArlS-ArlR is a regulator of virulence gene expression in *Staphylococcus aureus*. Mol. Microbiol. **41:**247–261.
16. **Gaskill, M. E., and S. A. Khan.** 1988. Regulation of the enterotoxin B gene in *Staphylococcus aureus*. J. Biol. Chem. **263:**6276–6280.
17. **Giraudo, A. L., A. L. Cheung, and R. Nagel.** 1997. The *sae* locus of *Staphylococcus aureus* controls exoprotein synthesis at the transcriptional level. Arch. Microbiol. **168:**53–58.
18. **Gordon, R. J., and F. D. Lowy.** 2008. Pathogenesis of methicillin-resistant *Staphylococcus aureus* infections. Clin. Infect. Dis. **46:**S350–S359.
19. **Horsburgh, M. J., J. L. Aish, I. J. White, L. Shaw, J. K. Lithgow, and S. J. Foster.** 2002. σ^B modulates virulence determinants expression and stress resistance: characterization of a functional *rsbU* strain of *Staphylococcus aureus* 8325-4. J. Bacteriol. **184:**5457–5467.
20. **Kaito, C., D. Morishita, Y. Matsumota, K. Kurokawa, and K. Sekimizu.** 2006. Novel DNA binding protein SarZ contributes to virulence in *Staphylococcus aureus*. Mol. Microbiol. **62:**1601–1617.
21. **Klevens, R. M., M. A. Morrison, J. Nadle, S. Petit, K. Gershman, S. Ray, L. H. Harrison, R. Lynfield, G. Dumyati, J. M. Townes, A. S. Graig, E. R. Zell, G. E. Fosheim, L. K. McDougal, R. B. Carey, and S. K. Fridkin.** 2007. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. JAMA **298:**1763–1771.
22. **Koenig, R. L., J. L. Ray, S. J. Maleki, M. S. Smeltzer, and B. K. Hurlburt.** 2004. *Staphylococcus aureus* AgrA binding to the RNAIII-*agr* regulatory region. J. Bacteriol. **186:**7549–7555.
23. **Kornblum, J., B. Kreiswirth, S. J. Projan, H. Ross, and R. P. Novick.** 1990. Agr: a polycistronic locus regulating exoprotein synthesis in *Staphylococcus aureus*, p. 403–420. *In* R. P. Novick (ed.), Molecular biology of the staphylococci. VCH Publishers, New York, NY.
24. **Kuroda, M., T. Ohta, I. Uchiyama, T. Baba, H. Yuzawa, I. Kobayashi, L. Cui, A. Oguchi, K.-I. Aoki, Y. Nagai, J. Lian, T. Ito, M. Kanamori, H. Matsumaru, A. Maruyama, H. Murakami, A. Hosoyama, Y. Mizutani-Ui, N. K. Takahashi, T. Sawano, R.-I. Inoue, C. Kaito, K. Sekimizu, H. Hirakawa, S. Kuhara, S. Goto, J. Yabuzaki, M. Kanehisa, A. Yamashita, K. Oshima, K. Furuya, C. Yoshino, T. Shiba, M. Hattori, N. Ogasawara, H. Hayashi, and K. Hiramatsu.** 2001. Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. Lancet **357:**1225–1240.
25. **Lee, C. Y., S. L. Buranen, and Z. H. Ye.** 1991. Construction of single copy integration vectors for *Staphylococcal aureus*. Gene **103:**101–105.
26. **Lee, C. Y.** 1992. Cloning of genes affecting capsule expression in *Staphylococcus aureus* strain M. Mol. Microbiol. **6:**1515–1522.
27. **Lowy, F.** 1998. *Staphylococcus aureus* infections. N. Engl. J. Med. **339:**520–532.
28. **Luong, T. T., S. W. Newell, and C. Y. Lee.** 2003. MgrA, a novel global regulator in *Staphylococcus aureus*. J. Bacteriol. **185:**3703–3710.
29. **Luong, T. T., P. M. Dunman, E. Murphy, S. J. Projan, and C. Y. Lee.** 2006. Transcription profiling of the *mgrA* regulon in *Staphylococcus aureus*. J. Bacteriol. **188:**1899–1910.
30. **Manna, A., and A. L. Cheung.** 2001. Characterization of *sarR*, a modulator of *sar* expression in *Staphylococcus aureus*. Infect. Immun. **69:**885–896.
31. **Manna, A. C., M. G. Bayer, and A. L. Cheung.** 1998. Transcriptional analysis of different promoters in the *sar* locus in *Staphylococcus aureus*. J. Bacteriol. **180:**3828–3836.
32. **Manna, A. C., and A. L. Cheung.** 2003. *sarU*, a *sarA* homolog, is repressed by SarT and regulates virulence genes in *Staphylococcus aureus*. Infect. Immun. **71:**343–353.
33. **Manna, A. C., S. S. Ingavale, M. Maloney, W. van Wamel, and A. L. Cheung.** 2004. Identification of *sarV* (SA2062), a new transcriptional regulator, is repressed by SarA and MgrA (SA0641) and involved in the regulation of autolysis in *Staphylococcus aureus*. J. Bacteriol. **186:**5267–5280.
34. **Manna, A. C., and A. L. Cheung.** 2006. Expression of SarX, a negative regulator of *agr* and exoproteins synthesis, is activated by MgrA in *Staphylococcus aureus*. J. Bacteriol. **188:**4288–4299.
35. **Manna, A. C., and A. L. Cheung.** 2006. Transcriptional regulation of the *agr* locus and the identification of DNA-binding residues of the global regulatory protein SarR in *Staphylococcus aureus*. Mol. Microbial. **60:**1289–1301.
36. **Manna, A. C., and B. Ray.** 2007. Regulation and characterization of *rot* transcription in *Staphylococcus aureus*. Microbiology **153:**1538–1545.
37. **McDevitt, D., P. Francois, P. Vandaux, and T. J. Foster.** 1994. Molecular characterization of the clumping factor (fibrinogen receptor) of *Staphylococcus aureus*. Mol. Microbiol. **11:**237–248.
38. **McNamara, P. J., K. C. Milligan-Monroe, S. Khalili, and R. A. Proctor.** 2000. Identification, cloning, and initial characterization of *rot*, a locus encoding a regulator of virulence factor expression in *Staphylococcus aureus*. J. Bacteriol. **182:**3197–3203.
39. **Morfeldt, E., K. Tegmark, and S. Arvidson.** 1996. Transcriptional control of the *agr*-dependent virulence gene regulator, RNAIII, in *Staphylococcus aureus*. Mol. Microbiol. **21:**1227–1237.
40. **Novick, R. P., H. F. Ross, S. J. Projan, J. Kornblum, B. Kreiswirth, and S. Moghazeh.** 1993. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J. **12:**3967–3977.
41. **Novick, R. P.** 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol. Microbiol. **48:**1429–1449.
42. **Oscarsson, J., C. Harlos, and S. Arvidson.** 2005. Regulatory role of proteins binding to the *spa* (protein A) and *sarS* (staphylococcal accessory regulator) promoter regions in *Staphylococcus aureus* NTCC8325-4. Int. J. Med. Microbiol. **295:**253–266.
43. **Pragman, A. A., J. M. Yarwood, T. J. Tripp, and P. M. Schlievert.** 2004. Characterization of virulence factor regulation by SrrAB, a two-component system in *Staphylococcus aureus*. J. Bacteriol. **186:**2430–2438.
44. **Queck, S. Y., M. Jameson-Lee, A. E. Villaruz, T. L. Bach, B. A. Khan, D. E. Sturdevant, S. M. Ricklefs, M. Li, and M. Otto.** 2008. RNAIII-independent target gene control by the *agr* quorum-sensing system: insight into the evolution of virulence regulation in *Staphylococcus aureus*. Mol. Cell **32:**150–158.
45. **Rechtin, T. M., A. F. Gillaspy, M. A. Schumacher, R. G. Brennan, M. S. Smeltzer, and B. K. Hurlburt.** 1999. Characterization of the SarA virulence gene regulator of *Staphylococcus aureus*. Mol. Microbiol. **33:**307–316.
46. **Rice, K., R. Peralta, D. Bast, J. de Azavedo, and M. J. McGavin.** 2001. Description of staphylococcus serine protease (*ssp*) operon in *Staphylococcus aureus* and nonpolar inactivation of *sspA*-encoded serine protease. Infect. Immun. **69:**159–169.
47. **Riggs, A. D., H. Suzuki, and S. Bourgeois.** 1970. Lac repressor-operator interaction. I. Equilibrium studies. J. Mol. Biol. **48:**67–83.
48. **Said-Salim, B., P. M. Dunman, F. M. McAleese, D. Macapagal, E. Murphy, P. J. McNamara, S. Arvidson, T. J. Foster, S. J. Projan, and B. N. Kreiswirth.** 2003. Global regulation of *Staphylococcus aureus* genes by Rot. J. Bacteriol. **185:**610–619.
49. **Schmidt, K. A., A. C. Manna, S. Gill, and A. L. Cheung.** 2001. SarT, a repressor of alpha-hemolysin in *Staphylococcus aureus*. Infect. Immun. **69:**4749–4758.
50. **Schmidt, K. A., A. C. Manna, and A. L. Cheung.** 2003. *sar*T influences the expression of *sarS* in *S. aureus*. Infect. Immun. **71:**5139–5148.
51. **Tamber, S., and A. L. Cheung.** 2008. SarZ promotes the expression of virulence factors and represses biofilm formation by modulating SarA and *agr* in *Staphylococcus aureus.* Infect. Immun. doi:10.1128/IAI.00859-08.
52. **Tegmark, K., A. Karlsson, and S. Arvidson.** 2000. Identification and characterization of SarH1, a new global regulator of virulence gene expression in *Staphylococcus aureus*. Mol. Microbiol. **37:**398–409.
53. **Trotonda, M. P., A. C. Manna, A. L. Cheung, I. Lasa, and J. R. Penades.** 2005. SarA controls Bap-dependent biofilm formation in *Staphylococcus aureus*. J. Bacteriol. **187:**5790–5798.
54. **Truong-Bolduc, Q. C., X. Zhang, and D. C. Hooper.** 2003. Characterization of NorR protein, a multifunctional regulator of *norA* expression in *Staphylococcus aureus*. J. Bacteriol. **185:**3127–3138.
55. **Truong-Bolduc, Q. C., and D. C. Hooper.** 2007. The transcriptional regulators NorG and MgrA modulate resistance to both quinolones and β-lactams in *S. aureus*. J. Bacteriol. **189:**2996–3005.
56. **Wang, L., M. Li, D. Dong, T. H. Bach, D. E. Sturdevant, C. Vuong, M. Otto, and Q. Gao.** 2008. SarZ is a key regulator of biofilm formation and virulence in *Staphylococcus epidermidis*. J. Infect. Dis. **197:**254–262.