INFECTION AND IMMUNITY, Jan. 2009, p. 419–428 Vol. 77, No. 1
0019-9567/09/$08.00+0 doi:10.1128/IAI.00859-08

SarZ Promotes the Expression of Virulence Factors and Represses Biofilm Formation by Modulating SarA and *agr* in *Staphylococcus aureus*▽

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Received 11 July 2008/Returned for modification 20 August 2008/Accepted 22 October 2008

Staphylococcus aureus **is a remarkably adaptable organism capable of multiple modes of growth in the human host, as a part of the normal flora, as a pathogen, or as a biofilm. Many of the regulatory pathways governing these modes of growth are centered on the activities of two regulatory molecules, the DNA binding protein SarA and the regulatory RNAIII effector molecule of the *agr* system. Here, we describe the modulation of these regulators and their downstream target genes by SarZ, a member of the SarA/MarR family of transcriptional regulators. Transcriptional and phenotypic analyses of a *sarZ* mutant demonstrated that the decreased transcription of *mgrA* and the *agr* RNAIII molecule was accompanied by increased transcription of *spa* (protein A) and downregulation of *hla* (alpha-hemolysin) and *sspA* (V8 protease) transcripts when compared to its isogenic parent. The decrease in protease activity was also associated with an increase in SarA expression. Consistent with an increase in SarA levels, the *sarZ* mutant displayed an enhanced ability to form biofilms. Together, our results indicate that SarZ may be an important regulator governing the dissemination phase of *S. aureus* infections, as it promotes toxin expression while repressing factors required for biofilm formation.**

Staphylococcus aureus is a gram-positive organism that colonizes the anterior nares of approximately 30% of the normal population. Infections can occur when there is a breach in the immune defenses of the host. Diseases caused by *S. aureus* vary, depending on the site of colonization, and can range from superficial skin lesions to invasive syndromes, such as pneumonia, endocarditis, osteomyelitis, and septicemia (3, 8). Adding to the seriousness of these infections is the propensity of *S. aureus* to form biofilms, which often leads to the establishment of chronic infections that are difficult to treat (12). The switching from the commensal, to the invasive, to the biofilm modes of growth is due in part to the complex repertoire of regulatory molecules that *S. aureus* uses to sense and respond to its environment.

The complex nature of the regulatory pathways governing virulence and biofilm formation in *S. aureus* arises from the fact that multiple regulators can activate or repress a single target gene. Detailed examination of the regulation of several virulence genes, such as *spa* (protein A), *hla* (alpha-hemolysin), and *sspA* (V8 protease), has revealed the following trends (3, 26–28, 36). First, gene expression occurs in a temporal fashion such that cell wall proteins and surface adhesins are expressed during the early, colonizing stages of infection, whereas toxins and secreted proteins are expressed later, during the tissue-damaging phase of disease. Second, the *agr* quorum-sensing system and the SarA family of DNA binding proteins form the cornerstone of virulence gene regulation in staphylococci. Together, these regulators control the expression of over a hundred genes that are involved in a myriad of cellular functions (11).

The *agr* system is comprised of two divergent transcripts (RNAII and RNAIII) that are activated in response to cell density. RNAII encodes a quorum-sensing two-component regulatory system that is activated by the autoinducing peptide. The RNAIII transcript, presumably activated by its cognate two-component regulatory system, is the *agr* effector molecule that activates the transcription of toxin genes such as *hla* and *sspA* and represses surface protein genes, such as *spa*, through its direct action or via intermediary regulators such as SarS, SarT, Rot, and SaeRS (14, 23, 25, 32).

The *sarA* gene encodes a DNA binding protein belonging to the SarA protein family of winged-helix transcriptional regulators (7). The SarA protein binds and activates the promoters of a number of genes, including *agr*, and represses other genes, such as *sspA* and *spa* (5, 9). Like *agr*, SarA also controls gene expression indirectly through its effect on other regulatory molecules. For example, the positive effect of SarA on *hla* is believed to be mediated in part by the repressor SarT (27).

Many members of the SarA family have been characterized and the majority of them have roles in controlling the expression of genes involved in virulence (7). One SarA homologue, SarZ, was previously reported to restore hemolysis in a mutant lacking that capability (18). We have now expanded that role to the general promotion of virulence through the activation of *agr* and repression of SarA. Additionally, we demonstrate a role for SarZ in the repression of biofilm formation, presumably through its effect on *sarA*. Together, these results suggest that SarZ plays an important role in the maintenance of active *S. aureus* infections.

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▽ Published ahead of print on 27 October 2008.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and reagents. The bacterial strains used in this study are listed in Table 1. *S. aureus* strains were routinely cultured on tryptic soy agar or broth (TSB) or 03GL broth (24). *Escherichia coli* strains were

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
S. aureus strains		
RN4220	Heavily mutagenized strain that accepts foreign DNA, *agr*-deficient	24
RN6390	Laboratory strain related to strain 8325-4	29
SH1000	8325-4 with *rsbU* restored	15
ALC6374	RN6390 Δ*sarZ*	This work
ALC6366	SH1000 Δ*sarZ*	This work
ALC6367	RN6390 Δ*sarZ*/*sarZ* complement	This work
ALC6368	RN6390 Δ*sarZ* with pEPSA5	This work
ALC6369	RN6390 Δ*sarZ* with pEPSA5::*sspA*	This work
ALC6370	RN6390 Δ*sarZ* with pEPSA5::*mgrA*	This work
ALC6410	RN6390 Δ*sarZ* Δ*sarA*	This work
ALC6409	RN6390 Δ*sarZ* with pRN6735 (*blaZ* promoter driving RNAIII)	This work
E. coli strain TOP10	General cloning strain	
Plasmids		
pMAD	*E. coli*-*S. aureus* shuttle vector containing a thermosensitve origin of replication, *bgaB*, Em^r Ap^r	1
pEPSA5	*E. coli*-*S. aureus* shuttle vector containing a xylose-inducible promoter, Ap^r Cm^r	13
pALC1484	*E. coli*-*S. aureus* shuttle vector derived from pSK236 containing the gfp_{uvr} gene, Ap^r Cm^r	17
pALC1540	*sarA* triple promoter driving the expression of gfp_{uvr} in pALC1484	21
pALC1435	*sarA* P1 promoter driving the expression of gfp_{uvr} in pALC1484	21
pALC1743	*agr* P3 promoter driving the expression of gfp_{uvr} in pALC1484	17
pALC1741	*spa* promoter driving the expression of gfp_{uvr} in pALC1484	19
pALC1740	*hla* promoter driving the expression of gfp_{uvr} in pALC1484	19
pALC2831	*sspA* promoter driving the expression of gfp_{uvr} in pALC1484	This work
pALC2566	*mgrA* promoter driving the expression of gfp_{uvr} in pALC1484	This work
pALC5448	pMAD containing a *sarA* deletion fragment generated by PCR	34
pRN6735	*blaZ* promoter driving the expression of promoterless RNAIII, Cm^r	35

grown on Luria-Bertani agar or broth. Antibiotics for plasmid selection and maintenance were used at the following concentrations: *S. aureus*, 2.5 μg/ml erythromycin and 10 μg/ml chloramphenicol; *E. coli*, 100 μg/ml ampicillin. All strains were grown in 18-mm borosilicate glass tubes at 37°C in an Excella E24 incubator (New Brunswick Scientific, Edison, NJ) with shaking at 250 rpm unless indicated otherwise. Bacterial growth was monitored by measuring the optical density at 650 nm (OD_{650}) on a Spectronic 20D+ spectrophotometer (Spectronic Analytical Instruments, Garforth, England). Early exponential, late exponential, and postexponential growth phases corresponded to OD readings of 0.7, 1.1, and 1.7, respectively, at 650 nm.

All chemicals and reagents were obtained from either Fisher or Sigma unless noted otherwise. Enzymes and reagents used for genetic manipulations were obtained from New England Biolabs.

Oligonucleotides and strain construction. A list of the oligonucleotides used in this study is available from the authors upon request. DNA isolation, electrophoresis, PCRs, transformation of *E. coli* and *S. aureus*, and other genetic manipulations were carried out according to standard laboratory protocols. Plasmid DNA from *E. coli* was first introduced into the heavily mutagenized, DNA restriction system-deficient *S. aureus* strain RN4220 prior to transfer into other *S. aureus* strains.

An unmarked, in-frame deletion of *sarZ* was constructed by employing a PCR splicing by overlap extension approach. Briefly, 1-kb regions up and downstream of the *sarZ* coding region were amplified using primers with complementary 9-bp overhangs. The resulting amplicons were used as the template to create a composite DNA fragment missing *sarZ*. This fragment was cloned into pMAD, a shuttle vector with a temperature-sensitive origin of replication in *S. aureus* (1). Through a series of temperature shifts from 30°C to 44°C, the *sarZ* deletion construct was obtained via a double-crossover event in the chromosome, replacing the native *sarZ* chromosomal region with the one containing the in-frame markerless deletion. A similar approach, wherein the native *sarZ* gene was crossed back into the chromosome of the *sarZ* mutant, was used to create the *sarZ*-complemented strain. The *sarA sarZ* double mutant was constructed by introducing the pMAD derivative pALC5448 into the *sarZ* mutant, allowing for the replacement of the native *sarA* gene with the mutant allele. Correct clones were identified by PCR and confirmed by sequencing.

The SspA- and MgrA-overproducing strains were created by amplifying the respective open reading frames and cloning them downstream of the xylose-inducible promoter of the *S. aureus* expression vector pEPSA5 (13). The respective promoter sequences of these genes were also amplified and cloned upstream of the gfp_{uvr} gene in the *E. coli*-*S. aureus* shuttle vector pALC1484 to create transcriptional fusions (17). The RNAIII-overproducing strain was constructed by introducing pRN6735 into the *sarZ* mutant.

Northern hybridizations. RNA was isolated from approximately 1.6×10^{11} CFU from early exponential phase (OD_{650}, 0.7), late exponential phase (OD_{650}, 1.1), or postexponential phase (OD_{650}, 1.7) cells grown in TSB. Cells were lysed in Trizol according to the manufacturer's protocol (Invitrogen, Irving, CA) using 0.1-mm silica-zirconia beads and a reciprocating shaker (BIO 101, Vista, CA). Ten micrograms of RNA was separated on a 1.5% agarose–0.66 M formaldehyde gel in 20 mM morpholinepropanesulfonic acid, 10 mM sodium acetate, 2 mM EDTA (pH 7) at 80 V for 3 h, transferred to a nylon membrane (Amersham HyBond XL; GE Healthcare, Piscataway, NJ) in 20× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7), and fixed by baking the membranes at 80°C for 2 h. Membranes were prehybridized in 5× SSC, 0.5% sodium dodecyl sulfate, and 5× Denhardt's solution (0.1% bovine serum albumin, 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone) for 4 h at 65°C. Purified DNA fragments were labeled with the random primed DNA labeling kit (Hoffmann-La Roche Inc., Nutley, NJ) and allowed to hybridize to the membranes overnight at 65°C. Posthybridization, the membranes were washed extensively and the bands were visualized by autoradiography. The relative intensities of the resulting bands were determined by using ImageJ (30). Data shown are representative of at least three hybridizations.

Green fluorescent protein (GFP)-promoter fusion analysis. Shuttle plasmids containing the promoter regions of various genes driving the expression of GFP_{uvr} (Table 1) were electroporated into the wild-type, *sarZ* mutant, and complemented strains by electroporation as previously described (31). Overnight cultures of the resulting strains were diluted 1:100 into fresh TSB and incubated at 37°C with shaking. Aliquots of the cultures were removed at specified time points, and their growth and fluorescence were monitored using an FL600 microplate fluorescence reader (BioTek Instruments, Winooski, VT). Experiments were repeated three times using four independently isolated clones read in triplicate. Error bars represent standard deviations of the means. Statistical significance was calculated using Student's *t* test.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western transfer. Cell lysates were prepared by harvesting approximately 1.6×10^{11} CFU from exponential-phase cells, resuspending the pellets in TEG buffer (25 mM

Tris-Cl, pH 8, 25 mM EGTA), and lysing the cells using 0.1-mm silica-zirconia beads and a reciprocating shaker. The proteins were separated from cellular debris by centrifugation at 12,000 × *g* for 5 min at 4°C and quantitated using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). An equivalent amount of protein (75 μg) was separated by electrophoresis on 12.5% acrylamide gels, transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA), and analyzed by Western blotting according to standard protocols. The primary antibody used was murine anti-SarA monoclonal antibody (1:1,000 dilution) (21), and the secondary antibody was donkey anti-mouse immunoglobulin G conjugated to horseradish peroxidase (1:10,000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). Binding of the antibodies to the membrane was detected using the ECL Western blotting detection system according to the manufacturer's instructions (GE Healthcare). Densitometry was performed using ImageJ (30).

Protease detection assays. Protease production was tested by spotting 2 μl of 10^{10} cells/ml onto agar plates containing 5% skimmed milk followed by incubation at 37°C for 24 h. Alternatively, proteins from 30 μl of culture supernatant from overnight bacterial cultures normalized to 10^{10} cells/ml were separated electrophoretically on 12.5% acrylamide gels containing 1 mg/ml gelatin. After electrophoresis, the gel was washed with gentle shaking in phosphate-buffered saline containing 2.5% Triton X-100 for 1 hour at room temperature and incubated overnight in protease detection buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$, 1 mM cysteine, 0.2% Triton X-100) at 37°C with shaking. The gel was then stained in Coomassie brilliant blue R-250 and destained according to standard procedures to visualize protease activity as zones of clearing on a blue background.

Biofilm formation and detachment assays. Biofilms were formed in 96-well polystyrene plates according to the method of Caiazza and O'Toole (4). Cells were inoculated at a concentration of 10^7 cells/ml into 100 μl of 66% TSB containing 0.2% glucose or a mixture of 2% xylose and 2% glucose and incubated for 16 h at 37°C. The culture supernatant and nonadherent bacteria were removed by decanting and then washing the wells three times with water. Biofilms were stained with 0.1% crystal violet for 15 min and washed again three times with water. The crystal violet was released from the adherent cells with absolute ethanol and quantified by reading its absorbance at 562 nm.

For detachment studies, preformed biofilms were treated with 100 μl 20 mM Tris-Cl (pH 7.5), 100 μg/ml protease K, or 0.14 U/ml DNase I (Roche) at 37°C for 2 h. Washing and staining of the biofilms were carried out as described above. Data shown are representative of three independent experiments with at least six replicates each. Error bars represent the standard deviations of the means. Statistical significance was calculated using Student's *t* test.

RESULTS

Transcriptional profile of the *sarZ* gene. SarZ was originally identified based on its homology with members of the SarA protein family of *S. aureus*. The *sarZ* locus, SA2174, is located near genes involved in the nitrate/nitrite utilization pathways in *S. aureus*. However, despite this proximity, *sarZ* does not appear to be in an operon with any of them. Directly downstream of *sarZ* is an uncharacterized gene that is predicted to encode a chaperone based on its sequence similarity to genes of the heat shock protein family bearing an alpha-crystallin domain (10).

We initially chose to study *sarZ* in the RN6390 background because this strain has been well-characterized and is accompanied by an assortment of regulatory mutants which have been evaluated previously (7). However, recognizing that RN6390 has a defective *rsbU*, which is required to activate the alternative sigma factor B, we conducted relevant phenotypic studies in strain SH1000, which has a restored *rsbU* (16) (see below).

The transcriptional profile of the *sarZ* gene in the wild-type strain RN6390 during various points of the *S. aureus* cell cycle was assessed by Northern blotting (Fig. 1B). The *sarZ* transcript appeared as two hybridizing bands, with a predominant species at 450 bp, the expected size of the monocistronic *sarZ*

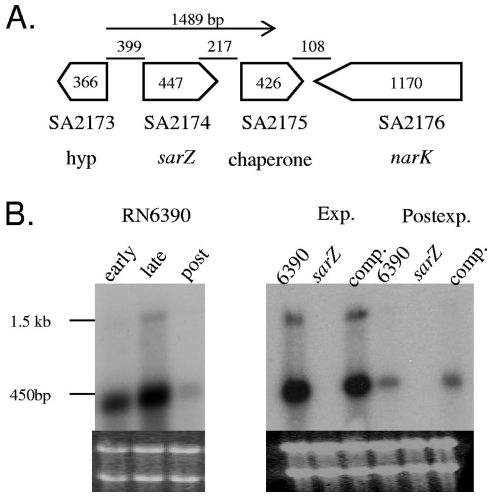

FIG. 1. Transcription of *sarZ* during the exponential and postexponential phases of growth. A. Genomic context of the *sarZ* gene. Numbers within the block arrows indicate the lengths of the genes in base pairs, and numbers above the arrows indicate the length of the intergenic regions, also in base pairs. B, left panel. Northern blot analysis of *sarZ* transcription in RN6390 during the early exponential (OD_{650}, 0.7), late exponential (OD_{650}, 1.1), and postexponential (OD_{650}, 1.7) phases of growth. Right panel. Northern blot analysis of *sarZ* transcription in the wild-type RN6390, *sarZ* mutant, and complemented *sarZ* mutant strains of *S. aureus* during late exponential and postexponential growth. Panels below the Northern blots show the 23S and 16S rRNAs, which served as the internal loading controls.

transcript, and a larger species at 1.5 kb, which likely corresponds to the cotranscription of *sarZ* with the putative chaperone SA2175 (Fig. 1A). As the growth cycle progressed, the level of *sarZ* transcript reached a maximal level during the late exponential phase (OD_{650}, 1.1). The level of the smaller *sarZ* transcript decreased during the postexponential phase (OD_{650}, 1.7), while the larger 1.5-kb transcript was not detectable. As expected, both transcripts were undetectable in the *sarZ* mutant, and the temporal pattern of *sarZ* transcription was restored upon complementation of the *sarZ* mutation.

Effect of a *sarZ* mutation on transcription of regulatory genes. Based on its similarity to SarA, we hypothesized that SarZ may be involved in regulation of the *S. aureus* virulence cascade. To test this hypothesis, the transcriptional profile of a *sarZ* deletion mutant was compared to that of the parental strain RN6390 and a complemented *sarZ* mutant strain. The levels of SarA in two independently isolated *sarZ* mutants were first assessed by immunoblotting. Compared to the parent strain, the two *sarZ* mutants had a two- to threefold increase in SarA protein level, suggesting that SarZ may have had a repressive effect on SarA expression (Fig. 2A). To confirm this, we analyzed *sarA* transcription in the *sarZ* mutant. The *sarA* locus is composed of three overlapping transcripts initiating from three distinct promoters, P1, P2, and P3 (21), that all encompass the *sarA* coding region. Northern blotting data revealed that the *sarZ* mutant demonstrated a moderate increase in the *sarA* P1 transcript during late exponential phase (OD_{650}, 1.1) compared with the parental and complemented strains (Fig. 2B, left panel). The level of the *sarA* P1 transcript in the mutant was similar to that of the parent during the postexponential phase. GFP-promoter fusion analysis, which reflects

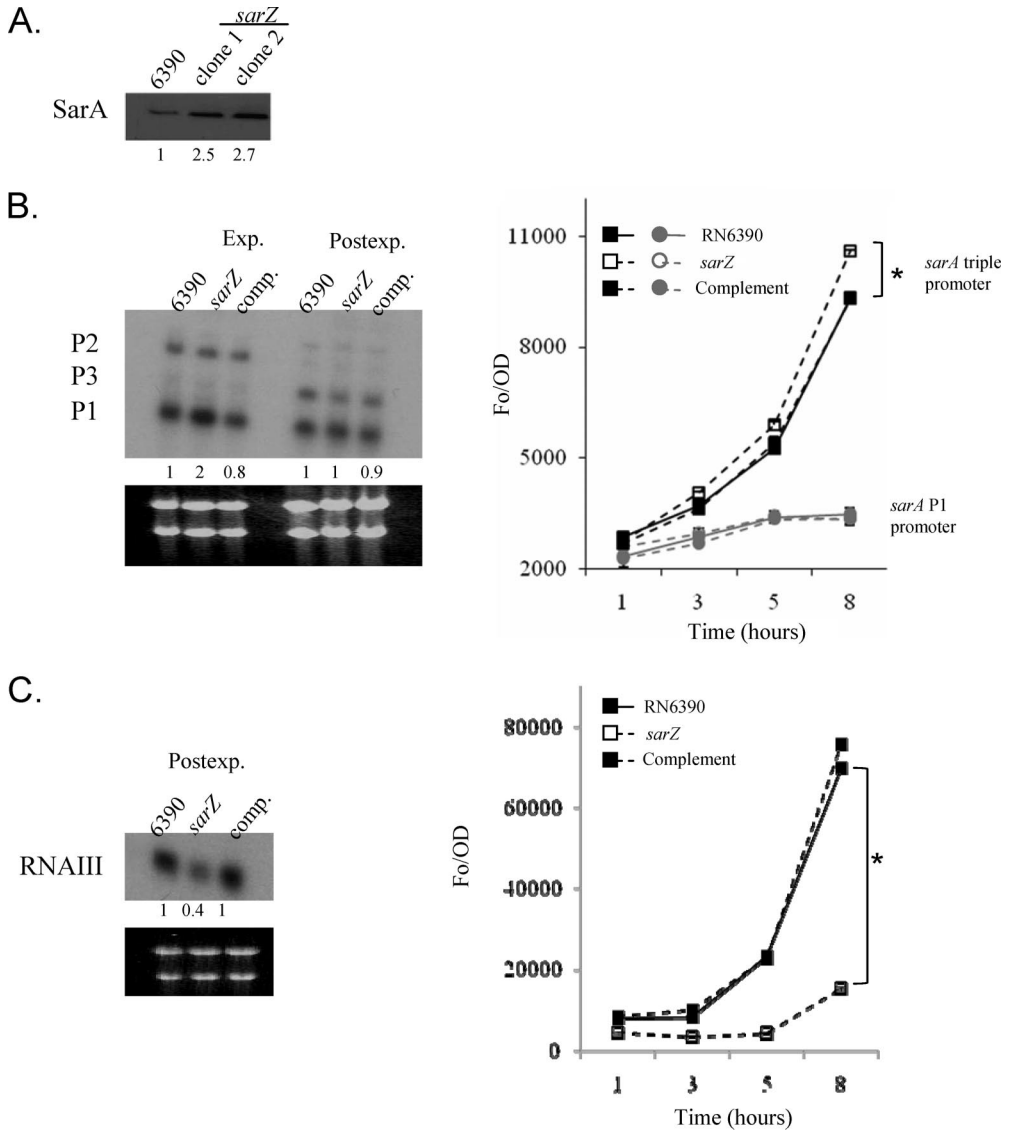

FIG. 2. Effect of *sarZ* on the expression of *sarA* and *agr*. A. Western blot analysis of SarA protein expression in the wild-type RN6390 and two independently isolated *sarZ* mutant clones of *S. aureus*. B and C, left panels. Northern blot analysis of *sarA* (B) and *agr* RNA III (C) transcription in the wild-type RN6390, *sarZ* mutant, and complemented *sarZ* mutant strains of *S. aureus*. Right panels. GFP-fusion analysis of the *sarA* triple promoter (black), *sarA* P1 promoter (gray), and *agr* RNAIII promoter activity in RN6390, *sarZ* mutant, and complemented strains of *S. aureus*. Numbers underneath the panels indicate the relative intensities or sum of the intensities of the bands as determined with Image J. Panels below the Northern blots show the 23S and 16S rRNAs, which served as the internal loading controls. *, $P < 0.01$, determined with Student's *t* test.

the cumulative promoter activity, indicated that the three strains had equivalent levels of P1 promoter activity during growth. When the collective strength of the native triple *sarA* promoter was investigated, its activity in the *sarZ* mutant was moderately, but significantly ($P = 0.001$), increased in comparison with the wild-type and complemented strains (Fig. 2B, right panel).

As SarA is a key regulator of *agr*, the level of *agr* RNAIII transcription was assessed in the *sarZ* mutant (Fig. 2C). Both Northern blotting and GFP-promoter fusion analyses indicated that the RNAIII transcript was strongly downregulated in the *sarZ* mutant in comparison with the wild-type and complemented strains.

Given that the levels of SarA were increased in a *sarZ*-deficient background and that SarA is an activator of *agr*, we reasoned that SarZ may have exerted its effect on *agr* independently of SarA. To evaluate this, we investigated the possibility that SarZ may have acted on downstream genes via SarR, a repressor of SarA and activator of *agr* (22). However, the level of *sarR* transcription was unchanged in the *sarZ* mutant in comparison to the wild type (data not shown), implying that SarZ likely activated *agr* independently of SarR as well as SarA.

Effect of a *sarZ* mutation on transcription of virulence genes. The repression of SarA and activation of *agr* by SarZ suggested that SarZ is an important player in the *S. aureus* virulence cascade. Therefore, the transcription of three virulence factor genes, controlled differentially by both SarA and *agr*, was ex-

amined in an attempt to delineate the regulatory network controlled by SarZ. The expression of the protein A gene, *spa*, is repressed by both SarA and *agr*. Northern blot analysis indicated that *spa* transcription was increased twofold in the *sarZ* mutant in comparison with the wild-type and complemented strains (Fig. 3A, left panel). This upregulation was confirmed by GFP-promoter fusion analysis. Given that SarA levels were increased in the *sarZ* mutant while RNAIII levels were decreased, these data indicated that SarZ probably represses *spa* expression by acting through a pathway mediated by *agr*. Indeed, provision of RNAIII in *trans* to the *sarZ* mutant halved the transcription of *spa*. As expected, removing *sarA*, a strong repressor of *spa*, from the *sarZ* mutant increased *spa* transcription by approximately 2.4-fold (Fig. 3A, center panel), indicating that these two regulators exert their effects upon *spa* independently of each other.

The transcription of *hla*, which encodes alpha-hemolysin, was moderately reduced in postexponentially growing *sarZ* mutant cells in comparison to the wild-type and complemented strains (0.7 versus 1) (Fig. 3B). The decrease in *hla* promoter activity was more evident when analyzing the GFP-promoter fusion activities during the growth cycle in the mutant versus the parent and complemented mutant strains. Deleting *sarA* from the *sarZ* mutant decreased *hla* transcription by fivefold, while providing RNAIII in *trans* increased *hla* transcription back to wild-type levels. Since *sarZ* activated *agr* while repressing *sarA*, and both of these activate *hla*, these data implied that the net effect of SarZ on *hla* transcription may be due to competing forces of *agr* and SarA.

The gene encoding the serine protease, *sspA*, is differentially regulated by *agr* and SarA. SarA represses the protease gene while *agr* activates it. As predicted, from the effect of *sarZ* upon SarA and *agr*, the transcription of *sspA* was markedly reduced (by approximately 10-fold) upon deletion of the *sarZ* gene (Fig. 3C, left panel). However, when *sarA* was deleted from the *sarZ* mutant or when RNAIII was provided in *trans*, the level of *sspA* transcription did not increase as expected (Fig. 3C, center panel). These data indicate that *sarZ* may activate *sspA* independently of both SarA and *agr*. Consistent with the transcriptional data, the *sarZ* mutant did not exhibit any detectable protease activity on either a skimmed milk agar plate or polyacrylamide gel containing gelatin (Fig. 3D).

Involvement of SarZ in biofilm formation. During the course of this work, it was noted that liquid cultures of the *sarZ* mutant settled out of solution more readily than either the wild-type or complemented strains, a phenotype that is frequently seen in biofilm-producing strains. Accordingly, a biofilm formation assay in a 96-well polystyrene plate format was performed; the *sarZ* mutant was found to have an increased ability to form biofilms in comparison to the parent and complemented mutant (Fig. 4A).

In *S. aureus*, the major biofilm exopolysaccharide, poly-*N*-acetylglucosamine (PNAG), is encoded by the *icaADBC* operon (12). However, Northern blot and GFP-promoter fusion analyses failed to demonstrate an alteration in the transcription of the *ica* genes in a *sarZ* mutant (data not shown), suggesting that the major constituent of biofilms formed by the *sarZ* mutant was not PNAG. Treatment of preformed biofilms with proteinase K promoted detachment of the biofilms, whereas treatment with DNase I did not. This result suggested that the biofilm-forming capacity of the *sarZ* mutant was likely protein mediated (Fig. 4B).

We also reasoned that the ability of the *sarZ* mutant to produce robust biofilms may have been due to the marked reduction in endogenous protease production. To confirm this hypothesis, the *sspA* gene was provided in *trans* to the mutant. The resultant strain was able to produce protease (Fig. 5, middle panel) and more importantly, exhibited reduced biofilm formation, to the level of the parental strain (Fig. 5, top and bottom panels).

Effect of SarZ on MgrA expression. Recently, unpublished data from our lab has demonstrated that another SarA homologue, MgrA, is involved in the repression of biofilm formation in an *ica*-independent manner, akin to that of SarZ. Hence, we sought to determine whether SarZ was involved in the regulation of MgrA. Both Northern and GFP-promoter fusion analyses demonstrated a noticeable decrease in *mgrA* transcriptional activity in the *sarZ* mutant compared with the parent, consistent with the notion that SarZ is an activator of *mgrA* (Fig. 6A). Provision of *mgrA* in *trans* under the control of an exogenous promoter was able to curtail biofilm formation of the *sarZ* mutant down to the levels of the wild-type strain (Fig. 6B). Although *sarZ* and *mgrA* are both positive regulators of *agr* and *sspA*, the effect of SarZ on biofilms likely occurred independently of *agr* and *sspA*, since provision of *mgrA* to the *sarZ* mutant in *trans* did not complement the transcription of these two genes, nor did it restore protease production in the *sarZ* mutant (Fig. 6C).

Role of SarZ in *S. aureus* strain SH1000. Our current understanding of virulence gene regulation in *S. aureus* is primarily through the study of laboratory strains derived from or closely related to RN6390 (16, 22, 26, 36). RN6390, however, has a deletion in *rsbU*, which encodes a phosphatase required for the activation of the alternative sigma factor B. As SigB has been implicated in the control of several virulence genes and biofilm formation, we sought to determine whether the effects we observed in the *sarZ* mutant of RN6390 were applicable to strain SH1000, a strain similar to RN6390 but with a restored *rsbU* (15). As observed with strain RN6390, *sarZ* was transcribed maximally in SH1000 during the late exponential phase of growth (Fig. 7A). Analysis of the levels of SarA, *agr*, and *mgrA* in two independently isolated *sarZ* mutants in SH1000 also mirrored results obtained with strain RN6390. In particular, the SarA protein level appeared to be repressed by SarZ, while transcription of *agr* and *mgrA* was activated by *sarZ* (Fig. 7B). In association with these regulatory changes, the *sarZ* mutant of SH1000 also demonstrated enhanced biofilm production compared with the parent (Fig. 7C). These data suggest that the regulatory profile and the biofilm-positive phenotype of the *sarZ* mutants occurred independently of the alternative sigma factor B.

DISCUSSION

In this study, we have shown by transcriptional and phenotypic analyses that SarZ plays an important role in the *S. aureus* virulence cascade (Fig. 8). Inactivation of *sarZ* in two strains of *S. aureus* resulted in elevated production of SarA in comparison to the respective parental strains. Using strain RN6390 as a model and building upon the accumulated regulatory data

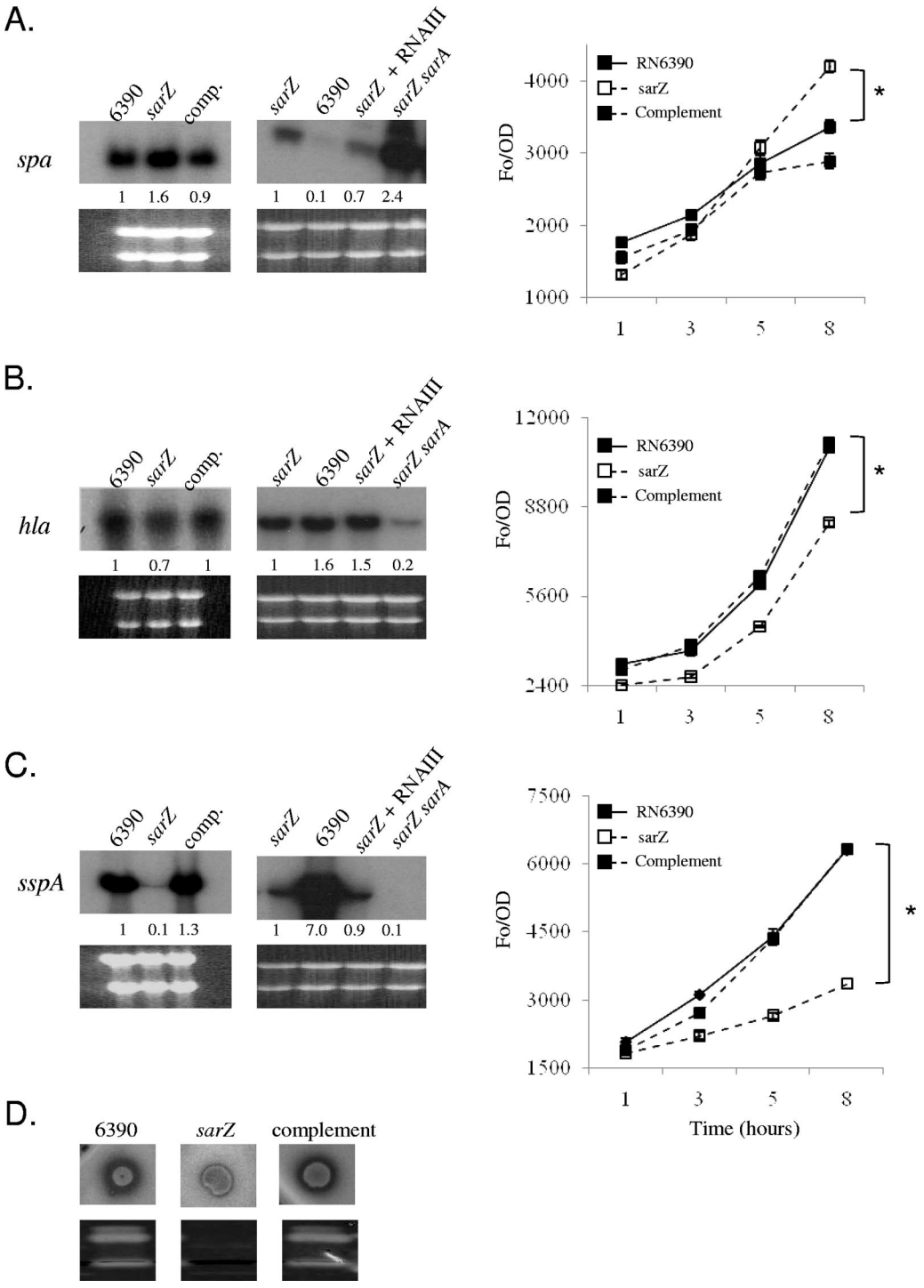

FIG. 3. Effect of *sarZ* on transcription of *spa*, *hla*, and *sspA*. (Left and center panels) Northern blot analyses of *spa* (A), *hla* (B), and *sspA* (C) in the RN6390 wild-type, *sarZ* mutant, and complemented *sarZ* mutant strains (left panel) and *sarZ* mutant, RN6390, *sarZ* mutant expressing RNAIII in *trans*, and *sarZ sarA* double mutant (center panel). RNA was harvested from late exponential phase (*spa*) or postexponential phase (*hla*, *sspA*) *S. aureus* cells. The lightness of the *spa* band in the middle portion of panel A was due to underexposure of the film, which allowed us to visualize the intensity of the *spa* band in the *sarZ sarA* double mutant. Numbers underneath the panels indicate the relative intensities of the bands as determined with ImageJ. Right panels. GFP-fusion analyses of the *spa* (A), *hla* (B), and *sspA* (C) promoter activities in RN6390, *sarZ* mutant, and complemented strains of *S. aureus*. *, $P < 0.01$ determined by Student's *t* test. D. Protease activity of the wild-type RN6390, *sarZ* mutant, and complemented *sarZ* mutant strains of *S. aureus*. Top: proteolysis on skimmed milk agar. Bottom: proteolysis on a gelatin-containing polyacrylamide gel.

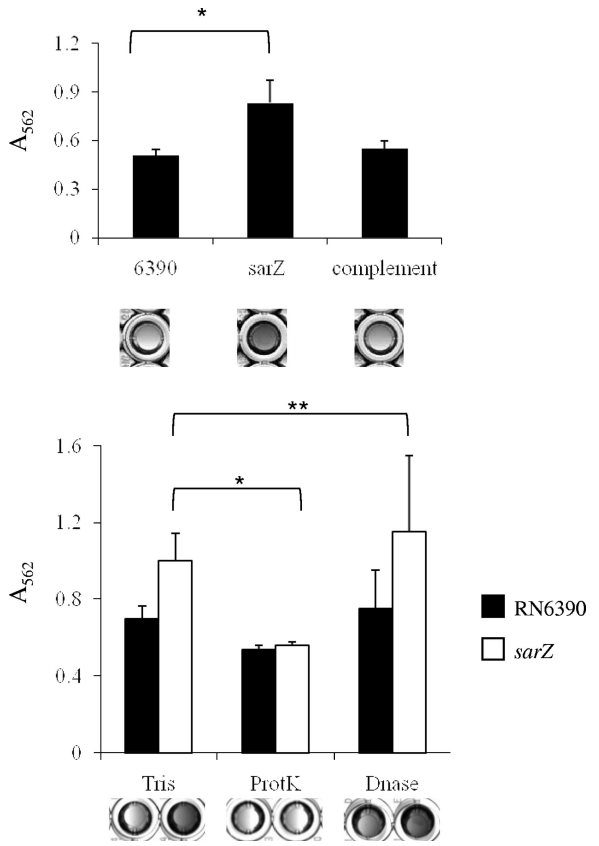

FIG. 4. A *sarZ* mutant hyperproduces biofilms rich in proteins. A. Quantitation of biofilm formation on 96-well polystyrene plates. *, $P < 0.01$ compared to the wild-type strain RN6390 determined by Student's *t* test. B. Detachment of biofilms formed by RN6390 and the *sarZ* mutant after treatment with proteinase K or DNase I for 2 h. *, $P < 0.01$ compared to the buffer control; **, $P > 0.05$ compared to the buffer control. Panels beneath the graphs show representative wells with the crystal violet staining phenotype of the biofilms.

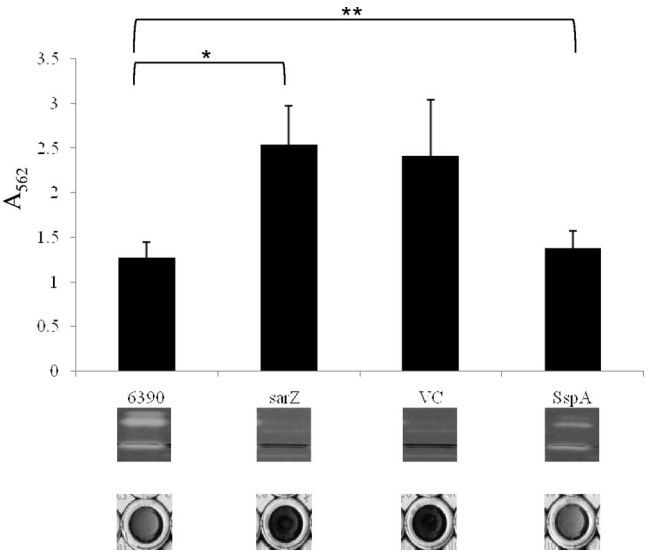

FIG. 5. Protease-mediated detachment of biofilms produced by a *sarZ* mutant. Top, biofilm formation of RN6390, the *sarZ* mutant, and the *sarZ* mutants containing either pEPSA5 (VC) or pEPSA5::*sspA* (SspA). Cells were grown on 66% TSB, 2% glucose, and 2% xylose to induce both biofilm formation and SspA production. *, $P < 0.01$ compared to the parental strain RN6390 as determined with Student's *t* test; **, $P > 0.05$ compared to the RN6390 parental strain. Middle panels, protease activity of the indicated strains on a gelatin-containing polyacrylamide gel. Bottom panels, representative wells showing the crystal violet staining phenotype of the biofilms.

on this strain, we found that a mutation in *sarZ* decreased *agr* transcription. Consistent with an *agr*-deficient phenotype, the transcription of *spa* was increased in the *sarZ* mutant, while the transcription of *hla* was decreased. Given that SarA upregulates *hla* and downmodulates *spa*, it is likely that the effects of SarZ on protein A and alpha-hemolysin gene expression are mediated primarily via *agr* rather than SarA (27). The drastic reduction of *sspA* transcription was first thought to be due to the augmented SarA expression and/or decreased *agr* RNAIII levels in the *sarZ* mutant. However, subsequent results with a *sarA sarZ* double mutant and a *sarZ* mutant overexpressing RNAIII demonstrated that the effect of *sarZ* upon *sspA* likely occurred independently of these two global regulators. Previous work by Kaito et al. (18) demonstrated the direct binding of SarZ to the *hla* and *agr* promoter, thus providing evidence that SarZ may directly regulate these genes. Whether this DNA binding activity also applies to the *sarA*, *sspA*, or *spa* promoters will require further study, especially in light of the finding that the binding of SarZ to DNA may be nonspecific (18).

Our observation that *sarZ* represses *sarA* (an activator of *agr*) and upregulates *agr* suggested that the activation of *agr* by SarZ occurred independently of SarA. We initially hypothesized that SarZ may act via SarR, a repressor of SarA and activator of *agr* (22). However, analysis of *sarR* transcription in a *sarZ* mutant did not reveal any reproducible changes with respect to the wild-type strain, suggesting that SarZ likely regulates *agr* and *sarA* independently of SarR. Based on the observed decrease in *mgrA* transcription in the *sarZ* mutant and its role in *agr* activation (16), MgrA was next examined as a potential intermediary between SarZ and *agr*. Provision of MgrA under the control of an exogenous promoter to the *sarZ* mutant repressed biofilm formation but was not sufficient in restoring the transcription of *agr* and its downstream target genes, *sspA* and *hla* (Fig. 6; *hla* data not shown), suggesting that SarZ or another factor controlled by SarZ was required for optimal *agr* activation. Detailed analysis of the binding activity of SarZ to the RNAIII promoter, alone or in conjunction with other regulators, such as SarA and MgrA, is required to fully appreciate the mechanistic details of *agr* regulation by SarZ.

The effect of *sarZ* on *sarA* expression is quite complex due to the intricacy of the triple promoters that drive the expression of *sarA* (5). Northern blot analysis revealed that *sarZ* may repress *sarA* due to its effect on the proximal P1 promoter during late exponential phase, which coincides with maximal *sarZ* transcription. GFP-promoter fusion analysis, however, suggested that the effect of SarZ upon *sarA* transcription may be attributable to the cumulative actions of SarZ on all three promoters throughout the growth cycle rather than on the *sarA* P1 promoter alone. Whether the discrepancy between *sarA* promoter activity and SarA protein expression levels in the

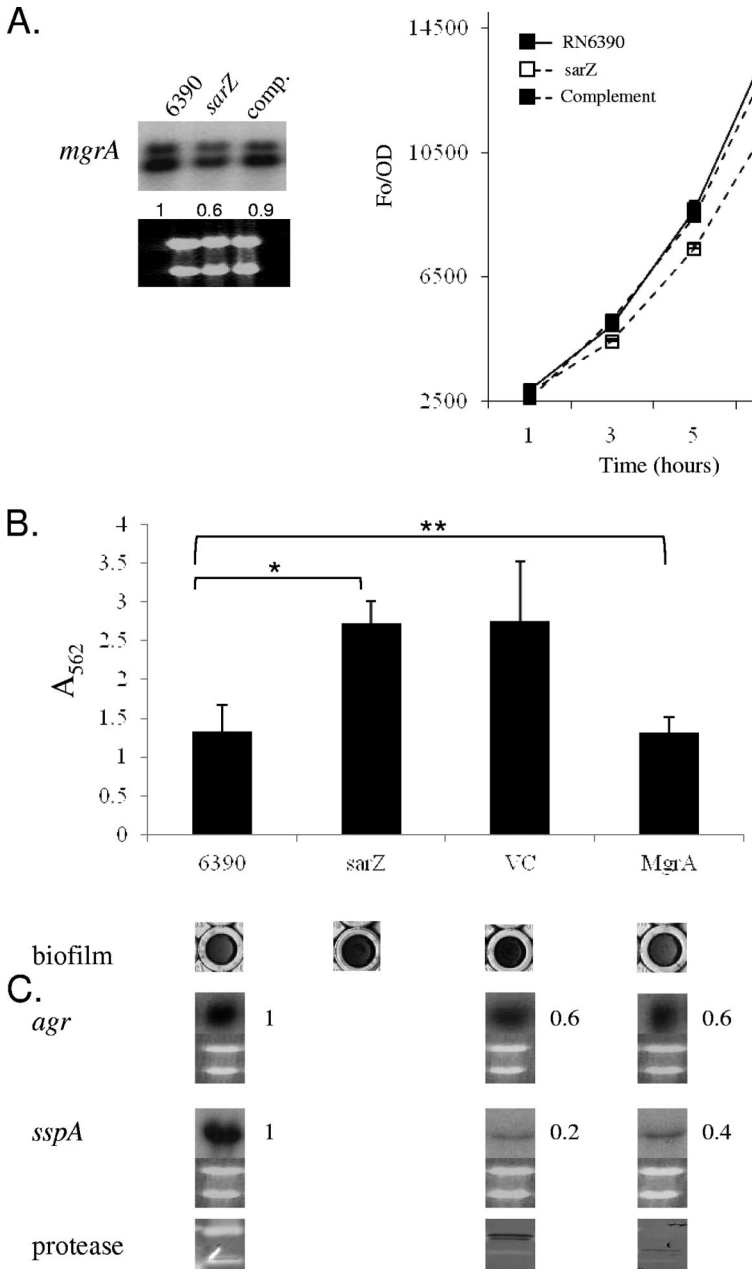

FIG. 6. Effect of *sarZ* on *mgrA*. A. Left panel. Northern blot analysis of *mgrA* transcription in RN6390, *sarZ* mutant, and complemented *sarZ* mutant strains of *S. aureus* during late exponential phase. Numbers underneath the panels indicate the relative intensities of the bands as determined with ImageJ. Right panel. GFP-fusion analysis of the *mgrA* promoter activity in RN6390, *sarZ* mutant, and complemented strains of *S. aureus*. *, $P < 0.01$ as determined by Student's *t* test. B. Top, biofilm formation of RN6390, the *sarZ* mutant, and the *sarZ* mutants containing pEPSA5 (VC) or pEPSA5::*mgrA* (MgrA). Cells were grown on 66% TSB, 2% glucose, and 2% xylose to induce both biofilm formation and MgrA production. *, $P < 0.01$ compared to RN6390; **, $P > 0.05$ compared to RN6390. Bottom panels, representative wells showing the crystal violet staining phenotype of the biofilms. C. Top two panels, transcription of *agr* and *sspA* in RN6390, *sarZ* mutant, and complemented *sarZ* mutant strains of *S. aureus* as determined by Northern blotting. Numbers beside the panels indicate the relative intensities of the bands as determined with ImageJ. Bottom panels, protease activity of the indicated strains on a gelatin-containing polyacrylamide gel.

sarZ mutant is attributable to an additional level of posttranscriptional control or occurs in conjunction with another regulator(s) remains to be defined. Indeed, both SarA and SarR can bind to and repress the *sarA* P1 promoter, thus indicating the complexity through which SarA protein expression may be controlled in *S. aureus* (6, 20).

As the *sarZ* mutant exhibited elevated SarA protein expression, we speculated that the *sarZ* mutant may have an enhanced biofilm phenotype. Previously, SarA has been shown to be essential to biofilm formation by activating the *ica* genes, which are required to synthesize PNAG, a major biofilm matrix component, and *bap*, encoding a protein adhesin found only in bovine isolates of *S. aureus* (33). However, the transcription of the *ica* genes was not altered in the *sarZ* mutant compared with

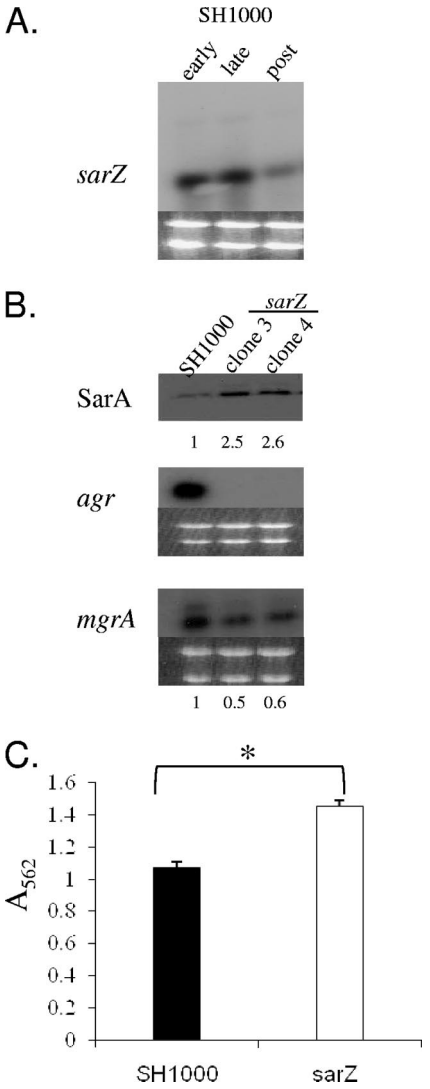

FIG. 7. Effects of *sarZ* are conserved in SH1000. A. Transcription of *sarZ* during the early exponential (OD_{650}, 0.7), late exponential (OD_{650}, 1.1), and postexponential (OD_{650}, 1.7) phases of growth. B. Effects of *sarZ* upon SarA, *agr*, and *mgrA* expression during late exponential (SarA, *mgrA*) and postexponential (*agr*) growth. Top panel, protein levels of SarA in the SH1000 wild-type strain and two independently isolated *sarZ* mutant clones of *S. aureus* as determined by Western blot analysis. Middle and bottom panels, Northern blot analysis of the transcription of *agr* RNAIII (middle) and *mgrA* (bottom) in the wild-type SH1000 strain and two independently isolated *sarZ* mutant clones of *S. aureus*. Numbers beneath the panels indicate the relative intensities of the bands as determined with ImageJ. C. Biofilm formation of SH1000 and an isogenic *sarZ* mutant in a 96-well polystyrene plate. *, $P < 0.05$ as determined by Student's *t* test.

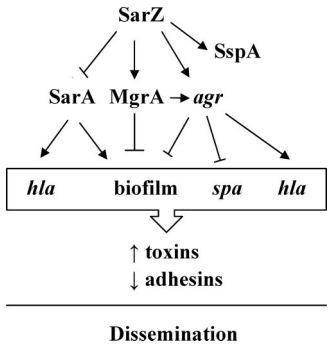

FIG. 8. Proposed mechanism of virulence gene regulation by SarZ. SarZ represses *sarA* and activates *mgrA*, leading to the downregulation of genes important to biofilm formation. Activation of *agr* by SarZ also leads to the upregulation of *hla* (alpha-hemolysin) and repression of *spa* (protein A). Additionally, SarZ activates the production of the SspA protease independently of both *agr* and *sarA*. These changes in gene expression result in a net decrease in surface adhesins and increase in toxins, which likely enable the bacteria to spread to new sites of infection.

the parental strain. Detachment studies with proteinase K and DNase I indicated that the major constituents of biofilms of *sarZ* mutants are likely proteins rather than a carbohydrate such as PNAG. Accordingly, there appear to be two plausible scenarios by which SarZ may repress biofilm formation in *S. aureus*: (i) by repressing surface adhesin expression via downregulation of SarA and (ii) through the activation of an inhibitory or detachment factor such as a protease. One likely candidate for a detachment factor was the V8 protease, SspA, since it was strongly repressed in the *sarZ* mutant. Provision of *sspA* in *trans* to the mutant restored the biofilm phenotype to the parental level. However, a recent publication as well as unpublished data from our lab have demonstrated that an *sspA* mutant alone does not result in an enhanced biofilm phenotype, contrary to what one would expect if SspA were a major detachment factor (2). Therefore, we conclude that while *sspA* is not essential for biofilm formation, high levels of the protease can contribute to biofilm detachment by acting as a general protease, much like proteinase K.

MgrA has also been described as a negative regulator of biofilm formation that acts in an *ica*-independent manner (34). Given the similarities between the biofilms of *sarZ* and *mgrA* mutants, we sought to determine whether SarZ controlled *mgrA* with respect to biofilm formation. Indeed, *mgrA* transcription was decreased in the *sarZ* mutant in comparison to the wild-type strain. Importantly, the enhanced biofilm phenotype of the *sarZ* mutant returned to near parental levels when *mgrA* was provided in *trans* via a plasmid with a xylose-inducible promoter. We also monitored *agr* and *sspA* transcription in this *sarZ* mutant derivative and determined that this MgrA-overproducing construct did not restore the transcription of these genes to the levels of the parental strain, RN6390. The lack of protease activity in this *sarZ* mutant derivative implied that increased expression of a surface adhesin(s), possibly via the downregulation of *mgrA* and/or *agr*, may account for the enhanced biofilm phenotype in the absence of SarZ. Whether the upregulation of SarA in the *sarZ* mutant contributes to further enhancement of biofilm formation via the expression of additional surface protein adhesin(s) remains to be determined.

Taken together, our results demonstrate that SarZ upregulates *agr* and represses SarA expression to promote the expression of virulence genes, such as *hla* and *sspA*. Additionally, the *sarZ* locus also controls *mgrA* and *agr* to repress biofilm formation. Repression of biofilm formation prevents the establishment of sessile bacterial communities and hence promotes active infections. In addition, concomitant expression of toxic

proteins, such as alpha-hemolysin and V8 protease in a *sarZ*-positive strain, would lead to tissue damage and promote the spread of bacteria to new infection sites. Accordingly, we propose that SarZ is an important regulator required for the maintenance and spread of active *S. aureus* infections.

ACKNOWLEDGMENTS

This work was supported in part by NIH grant AI37142 to A.L.C. We thank Niles Donegan for critical reading of the manuscript.

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Editor: A. Camilli