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SarZ promotes the expression of virulence factors and represses biofilm formation by modulating SarA and *agr* in *Staphylococcus aureus*

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ABSTRACT

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 RNA III 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 down-regulation 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.

INTRODUCTION

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 (TCRS) that is activated by the autoinducing peptide, AIP. The RNAIII transcript, presumably activated by its cognate TCRS, 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.

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 Trypic Soy agar/broth or 03GL broth (24). *E. coli* strains were grown on Luria Bertani agar/broth. Antibiotics for plasmid selection and maintenance were used at the following concentrations; *S. aureus*- 2.5 µg/mL erythromycin, 10 µg/mL chloramphenicol, *E. coli*- 10 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) shaking at 250 RPM unless indicated otherwise. Bacterial growth was monitored by measuring the optical density (OD) at 650nm on a Spectronic 20D+ spectrophotometer (Spectronic Analytical Instruments, Garforth, England). Early exponential, late-exponential, and post-exponential 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, polymerase chain reactions (PCR), 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 SOEing approach. Briefly, 1 kb regions up and downstream of the *sarZ* coding region were amplified using primers with complementary 9 base pair 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 cross-over 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 over-producing 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 x 10^{11} CFU from early exponential (OD650 = 0.7), late exponential (OD650 = 1.1), or post-exponential (OD650 = 1.7) phase 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-0.66M formaldehyde gel in 20 mM morpholinepropanesulfonic acid, 10 mM sodium acetate, 2 mM EDTA (pH 7) at 80 V for 3 hours, transferred to a nylon membrane (Amersham HyBond XL, GE Healthcare, Piscataway, NJ) in 20X SSC (3M NaCl, 0.3 M sodium citrate, pH 7) and fixed by baking the membranes at 80°C for 2 hours. Membranes were prehybridized in 5X SSC, 0.5% sodium dodecyl sulfate, and 5X Denhardt's solution (0.1% bovine serum albumin, 0.1% Ficoll-400, 0.1% polyvinylpyrolidone) for 4 hours 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. Post-hybridization, 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.

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, 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 mean. Statistical significance was calculated using the Student's t-test.

SDS-PAGE and Western transfer

Cell lysates were prepared by harvesting approximately 1.6 x 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 x *g* for 5 minutes at 4°C and quantitated using the BCA Protein Assay lit (Pierce, Rockford, IL). An equivalent amount of protein (75 µg) was separated by electrophoresis on 12.5% acrylamide gels, transferred onto PVDF membranes (Polyvinylidene fluoride, Millipore, Billerica, MA) and analyzed by Western blotting according to standard protocols. The primary antibody used was murine anti-SarA monoclonal antibody (1:1000 dilution, 21) and the secondary antibody was donkey anti-mouse IgG 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 hours. 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 one 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 66% TSB containing 0.2% glucose or a mixture of 2% xylose and 2% glucose and incubated for16 hours at 37°C. The culture supernatant and non-adherent bacteria were removed by decanting and then washing the wells three times with water. Biofilms were stained with 0.1% crystal violet for 15 minutes, and washed again 3 times with water. The crystal violet was released from the adherent cells with absolute ethanol and quantified by reading its absorbance at 562nm.

For detachment studies, pre-formed biofilms were treated with 100 µL 20mM Tris-Cl (pH 7.5), 100 µg/mL protease K, or 0.14 U/mL DNase I (Roche) at 37°C for 2 hours. Washing and staining of the biofilms were carried out as described above. Data shown are representative

of 3 independent experiments with at least 6 replicates each. Error bars represent the standard deviation of the mean. Statistical significance was calculated using the 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 with 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, and 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 blots (Figure 1B). The *sarZ* transcript appeared as two hybridizing bands, with a predominant species at 450 bp, the expected size of the monocistronic *sarZ* transcript, and a larger species at 1.5 kb, which likely corresponds to the co-transcription of *sarZ* with the putative chaperone SA2175 (Figure 1A). As the growth

cycle progressed, the level of *sarZ* transcript reached a maximal level during the late exponential phase (OD650 = 1.1). The level of the smaller *sarZ* transcript decreased during the post-exponential phase (OD650 = 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 the 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 immunoblots. Compared to the parent strain, the two *sarZ* mutants had a two- to three-fold increase in SarA protein level, suggesting that SarZ may have had a repressive effect on SarA expression (Figure 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 blot data revealed that the *sarZ* mutant demonstrated a moderate increase in the *sarA* P1 transcript during late exponential phase (OD650 = 1.1) as 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 post-exponential phase. GFP-promoter fusion analysis, which reflects 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 (Figure 2B, right panel).

As SarA is a key regulator of *agr*, the level of *agr* RNAIII transcription was assessed in the *sarZ* mutant (Figure 2C). Both Northern blot and GFP-promoter fusion analysis indicated that the RNAIII transcript, the *agr* effector molecule, was strongly down-regulated 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 upon 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 the transcription of virulence genes

The repression of SarA and activation of *agr* by SarZ suggested that SarZ was 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 examined 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 two-fold in the *sarZ* mutant in comparison with the wild-type and complemented strains (Figure 3A, left panel). This up-regulation 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 (Figure 3A centre 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 post-exponentially growing *sarZ* mutant cells in comparison to the wild-type and complemented strains (0.7 vs. 1, Figure 3B). The decrease in *hla* promoter activity was more evident by 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 5 fold 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) on deletion of the *sarZ* gene (Figure 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 (Figure 3C, centre 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 (Figure 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 *sar*Z mutant was found to have an increased ability to form biofilms in comparison to the parent and complemented mutant (Figure 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 (Figure 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 (Figure 5, middle panel) and more importantly, exhibited reduced biofilm formation, to the level of the parental strain (Figure 5, top and bottom panels).

Effect of SarZ on MgrA expression

Recently, unpublished data from our lab has demonstrated that another SarA homologue, MgrA, was 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 as compared with the parent, consistent with the notion that SarZ is an activator of *mgrA* (Figure 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 (Figure 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 (Figure 6C).

Role of SarZ in *S. aureus* strain SH1000

Our current understanding of virulence gene regulation in *S. aureus* is due 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 (Figure 7A). Analysis of the levels of SarA, *agr*, and *mgrA* in two independently isolated *sarZ* mutants in SH1000 also mirrored results obtained from strain RN6390. In particular, the SarA protein level appeared to be repressed by SarZ, while transcription of *agr* and *mgrA* was activated by *sarZ* (Figure 7B). In association with these regulatory changes, the *sarZ* mutant of SH1000 also demonstrated enhanced biofilm production as compared with the parent (Figure 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 (Figure 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 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 up-regulates *hla* and down-modulates *spa*, it is likely that the effect of SarZ on protein A and alpha-hemolysin gene expression was 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 over expressing 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 non-specific (18).

Our observation that *sarZ* represses *sarA* (an activator of *agr*) and up-regulates *agr* suggested that the activation of *agr* by SarZ occurred independently of SarA. We initially hypothesized that SarZ may have acted 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* (Figure 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 and/or in conjunction with other regulators such as SarA and MgrA are 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 have been attributed 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 *sarZ* mutant are attributable to an additional level of post-transcriptional control or in conjunction with other 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 as compared with 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 appeared to be two plausible scenarios by which SarZ may repress biofilm formation in *S. aureus*: (a) by repressing surface adhesin expression via down-regulation of SarA and/or (b) 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 was 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 over-producing 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 surface adhesin(s), possibly via the down regulation 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 adhesion(s) remains to be determined.

Taken together, our results demonstrate that SarZ up-regulates *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. Repressing 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.

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TABLES

Table 1. Bacterial strains and plasmids used in this study

S. aureus	**Description**	**Reference**
RN4220	Heavily mutagenized strain that accepts foreign DNA, *agr*-	(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 pEPSA*5*	This work
ALC6369	RN6390 Δ*sarZ* with pEPSA*5*::*sspA*	This work
ALC6370	RN6390 Δ*sarZ* with pEPSA*5*::*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		
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 deriviatized 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)

FIGURE LEGENDS

Figure 1. Transcription of *sarZ* during the exponential and post-exponential phases of growth. A. Genomic context of the *sarZ* gene. Numbers within the block arrows indicate the lengths of the genes in base pairs, numbers above the arrows indicate the length of the intergenic regions in also in base pairs. **B.** Left panel. Northern blot analysis of *sarZ* transcription in RN6390 during the early (OD_{650} = 0.7), late (OD_{650} = 1.1), and post (OD_{650} = 1.7) exponential 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 post-exponential growth. Panels below the Northern blots show the 23S and 16S which served as the internal loading control.

Figure 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 (grey), 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 by Image J. Panels below the Northern blots show the 23S and 16S rRNA which served as the internal loading control. * $p < 0.01$ as determined by the Student's t-test.

Figure 3. Effect of *sarZ* on the transcription of *spa*, *hla* and *sspA*. Left and centre panels. Northern blot analyses of *spa* (**A.**), *hla* (**B.**), and *sspA* (**C.**) in the RN6390 wild-type, *sarZ* mutant, complemented *sarZ* mutant strains (left panel); *sarZ* mutant, RN6390, *sarZ* mutant expressing RNAIII *in trans* and *sarZ sarA* double mutant (centre panel). RNA was harvested from late-exponentially (*spa*) or post-exponentially (*hla*, *sspA*) growing *S. aureus*. The lightness of the *spa* band in the middle panel of Fig. 3A was due to underexposure of the film to allow 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 by Image J. 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*. * $p < 0.01$ as determined by the Student's t-test. **D.** Protease activity of the wild-type RN6390, *sarZ* mutant, and complemented *sarZ* mutant strains of *S. aureus*. Top panel; proteolysis on skimmed milk agar. Lower panel; proteolysis on a gelatin-containing polyacrylamide gel.

Figure 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 as determined by the Student's t-test. **B.** Detachment of biofilms formed by RN6390 (black) and the *sarZ* mutant (white) after treatment with Proteinase K or DNase I for 2 hours. * $p < 0.01$ 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.

Figure 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 pEPSA*5* (VC), or pEPSA*5*::*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 by the 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.

Figure 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 by Image J. 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 the Student's t-test. **B.** Top, biofilm formation of RN6390, the *sarZ* mutant, and the *sarZ* mutants containing pEPSA*5* (VC) or pEPSA*5*::*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 blot. Numbers beside the panels indicate the relative intensities of the bands as determined by Image J. Bottom panels, protease activity of the indicated strains on a gelatin-containing polyacrylamide gel.

Figure 7. Effects of *sarZ* are conserved in SH1000. A. Transcription of *sarZ* during the early (OD_{650} = 0.7), late (OD_{650} = 1.1), and post (OD_{650} = 1.7) exponential phases of growth. **B.** Effect of *sarZ* upon SarA, *agr*, and *mgrA* expression during late (SarA, *mgrA*) and post-exponential (*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 by Image J. **C.** Biofilm formation of SH1000 and an isogenic *sarZ* mutant in a 96 well polystyrene plate. * $p < 0.05$ as determined by the Student's t-test.

Figure 8. Proposed mechanism of virulence gene regulation by SarZ. SarZ represses *sarA* and activates *mgrA*, leading to the down regulation of genes important to biofilm formation. Activation of *agr* by SarZ also leads to the up-regulation 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.

Figure 1.

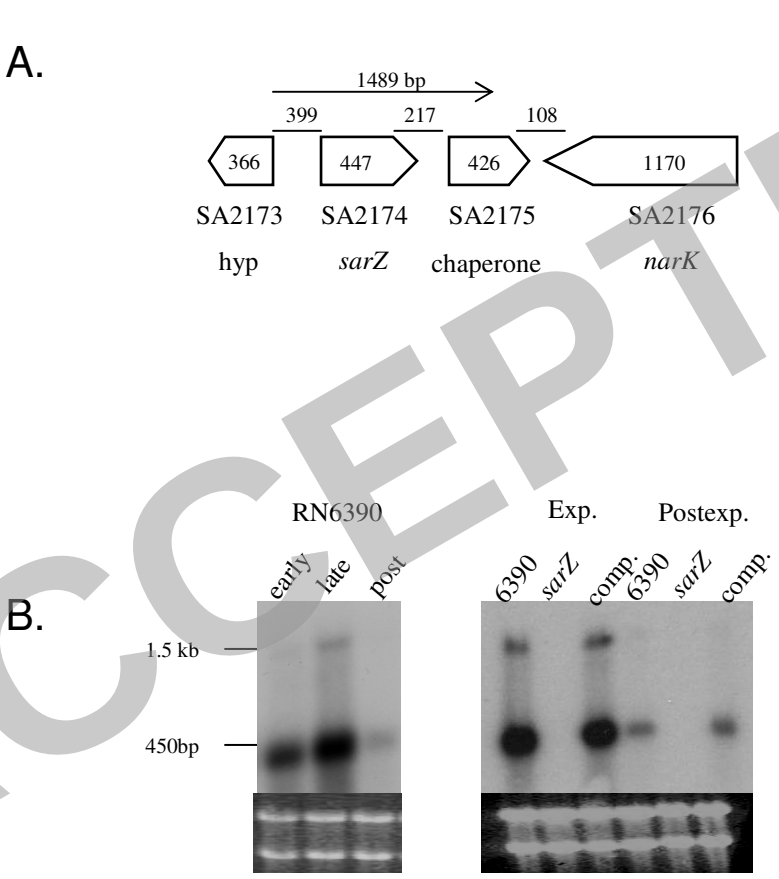
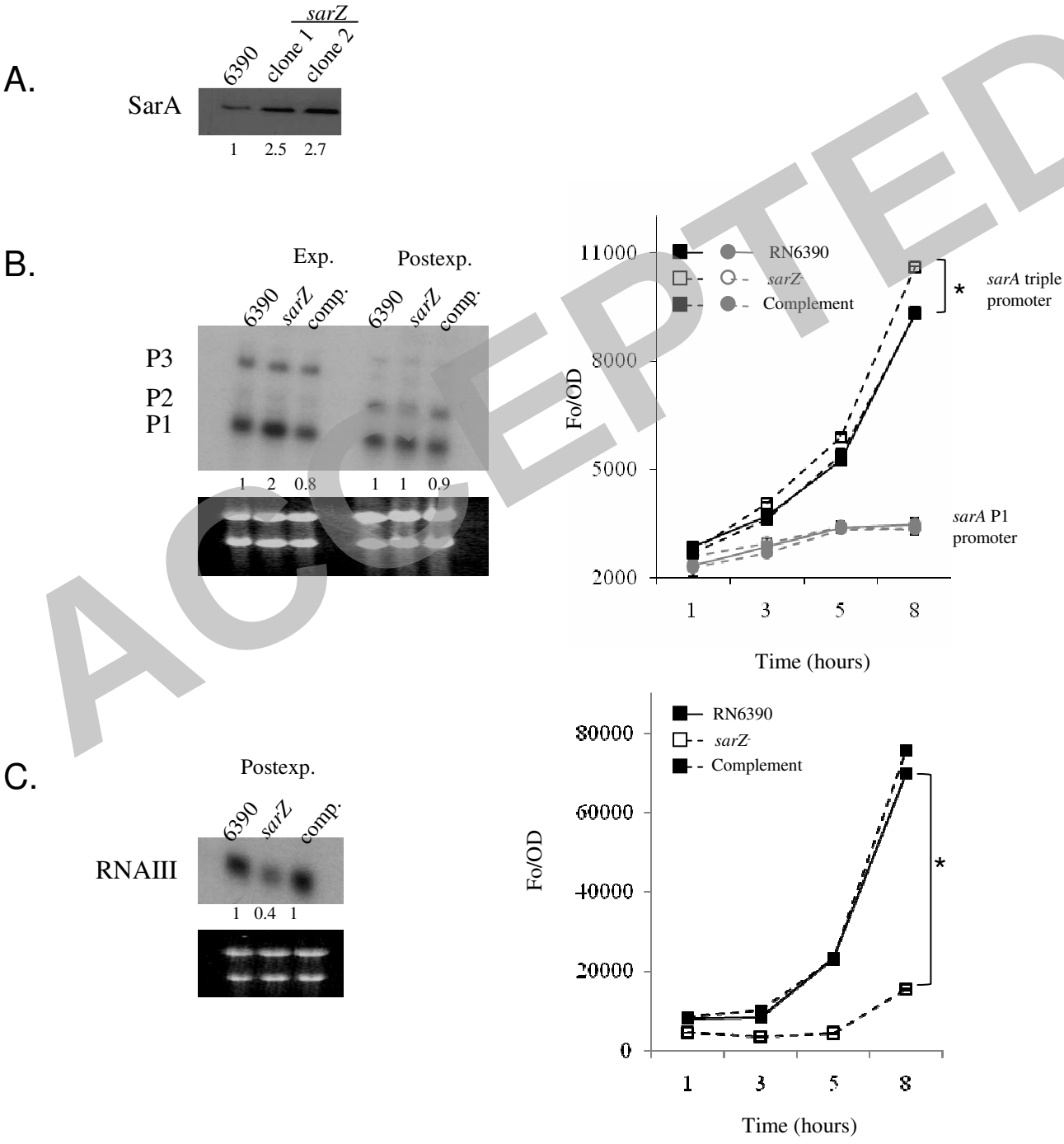


Figure 2.



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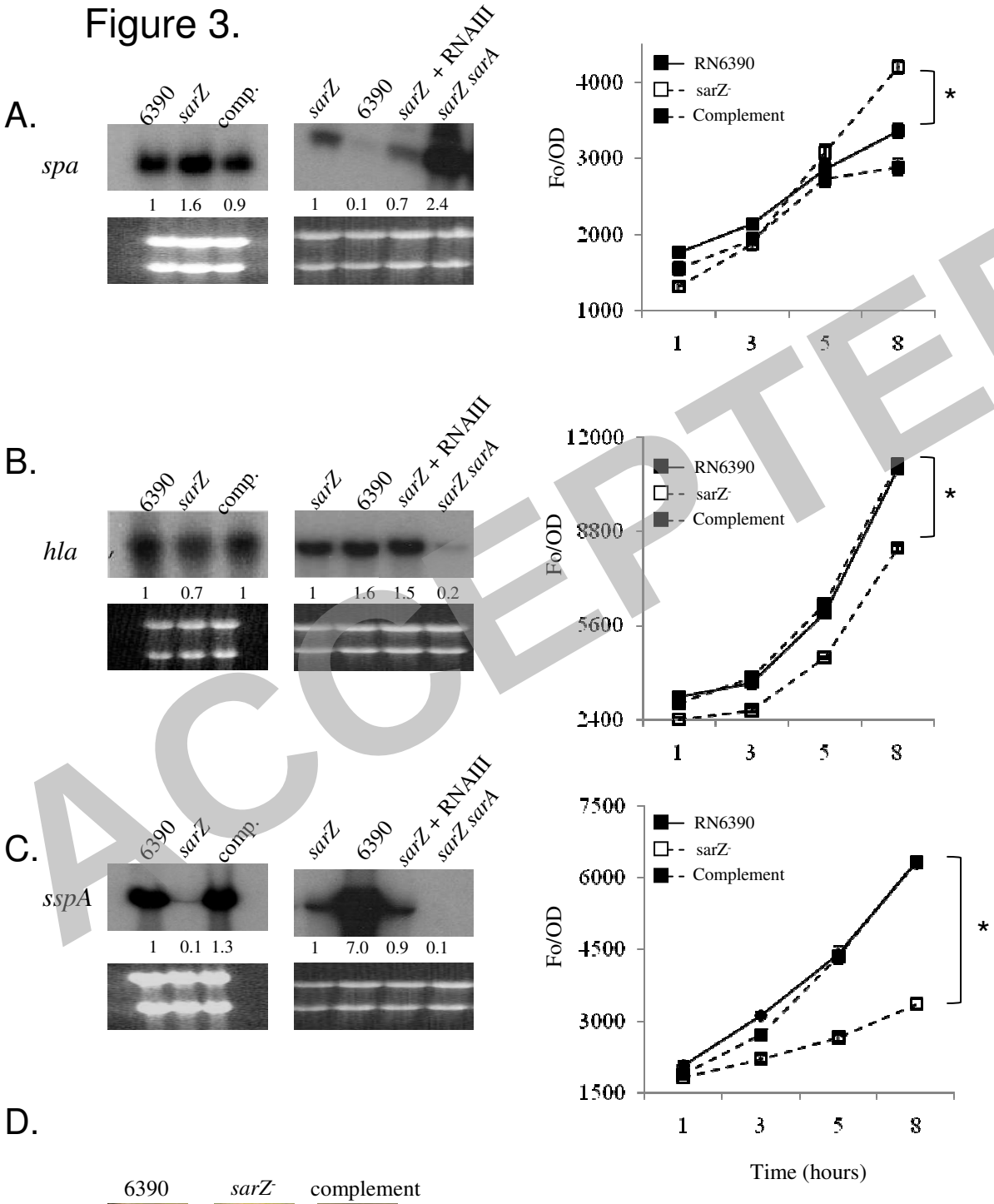

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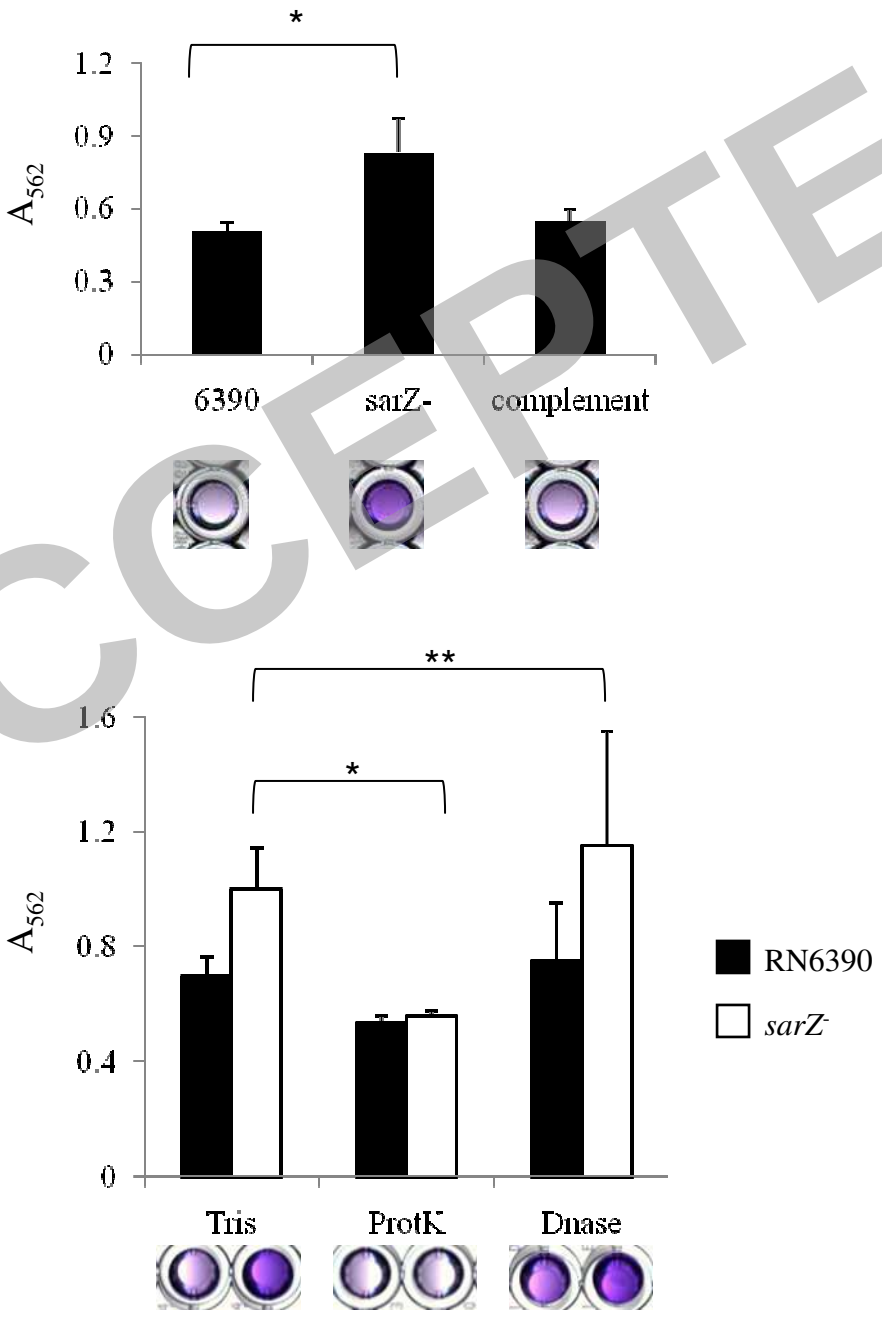

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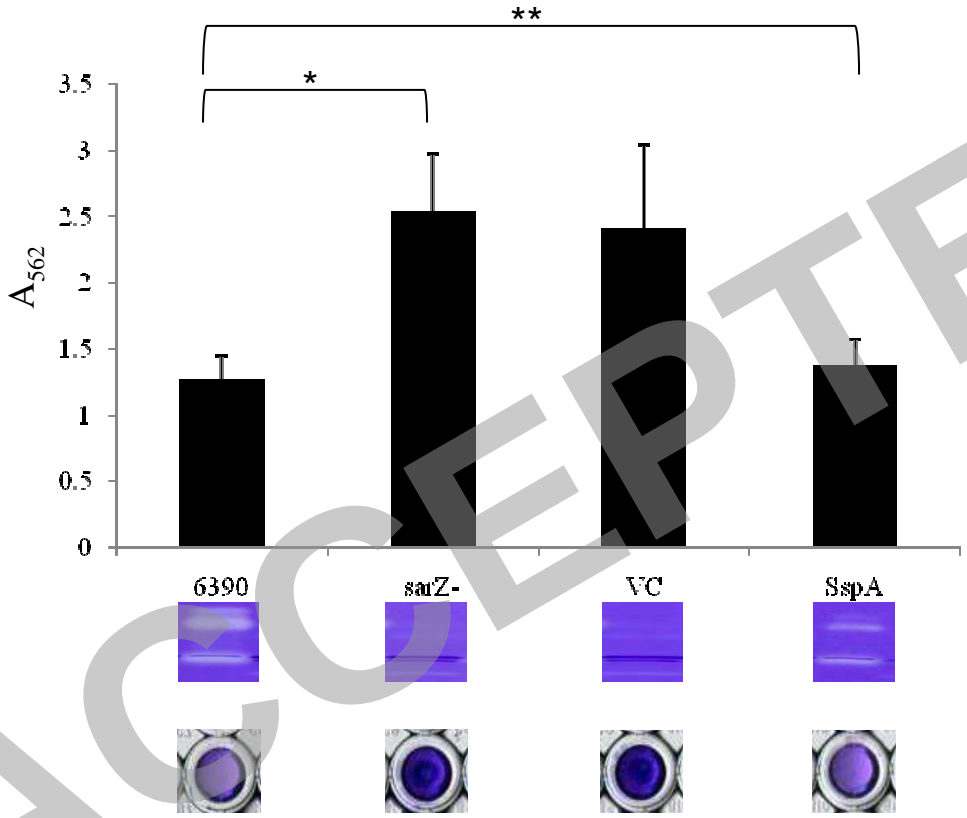

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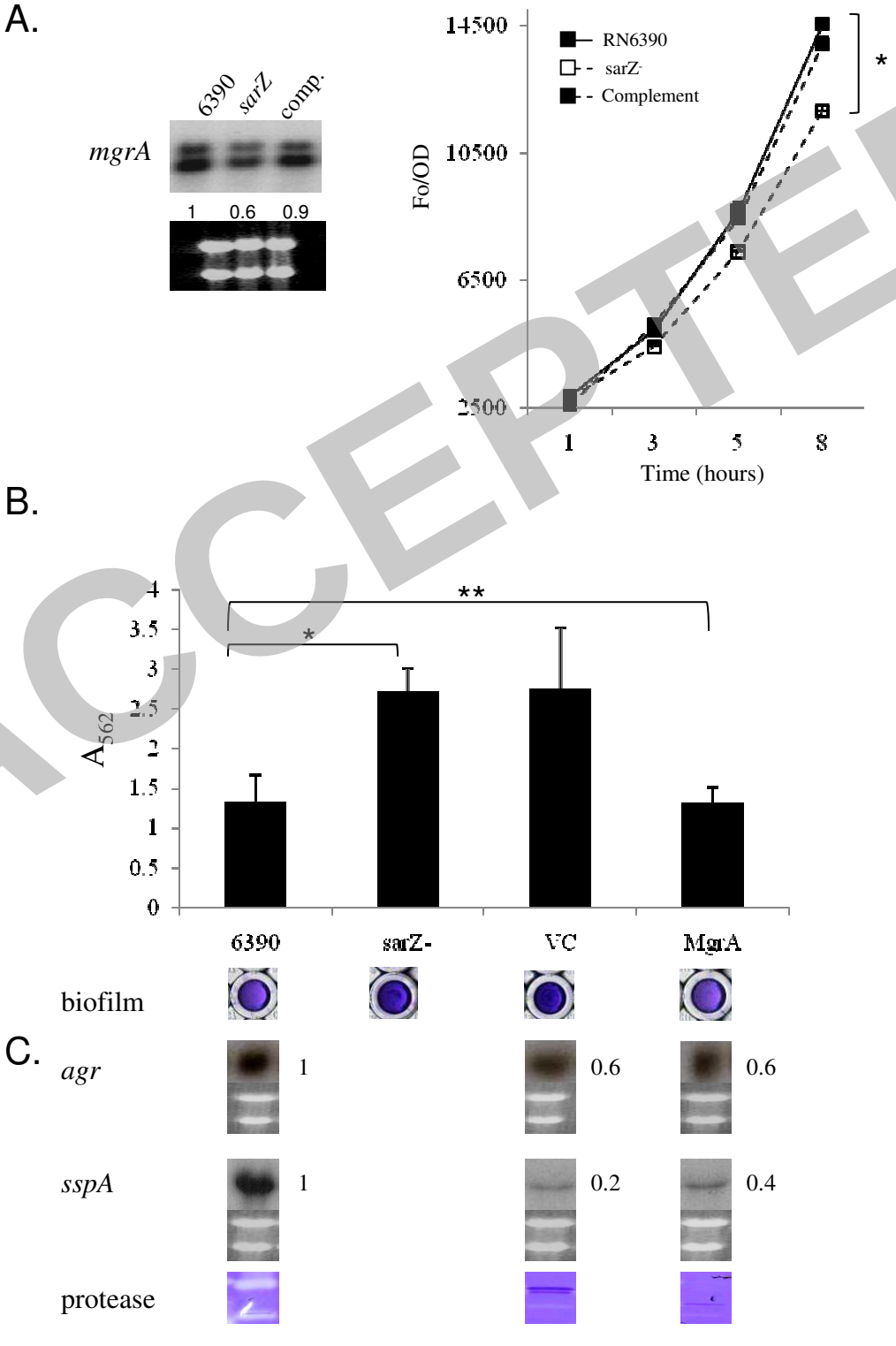

Figure 7.

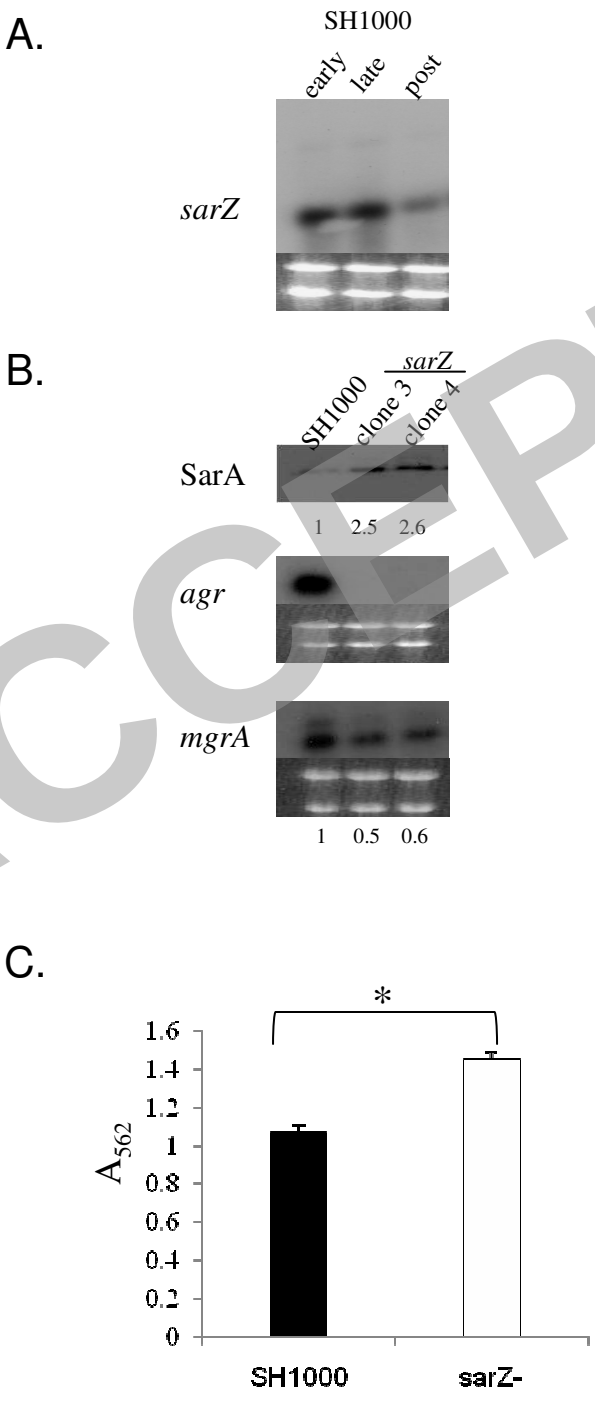

Figure 8.