

The Staphylococcus-Specific Gene *rsr* Represses *agr* and Virulence in *Staphylococcus aureus*[∇]

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The expression of virulence factors in *Staphylococcus aureus* is tightly coordinated by a vast network of regulatory molecules. In this report, we characterize a genetic locus unique to staphylococci called *rsr* that has a role in repressing two key virulence regulators, *sarR* and *agr*. Using strain SH1000, we showed that the transcription of virulence effectors, such as *hla*, *sspA*, and *spa*, is altered in an *rsr* mutant in a way consistent with *agr* upregulation. Analysis of RNAIII expression of the *agr* locus in *rsr* and *rsr-sarR* mutants indicated that *rsr* likely contributes to *agr* expression independently of SarR. We also provide evidence using a murine model of *S. aureus* skin infection that the effects mediated by *rsr* reduce disease progression.

Staphylococcus aureus is a leading cause of hospital- and community-acquired infections in the United States (8, 18). Its ability to colonize a variety of sites gives rise to a number of disease states ranging from superficial skin and soft tissue infections to pneumonia, endocarditis, osteomyelitis, and septic shock. Disease progression and severity are mediated by a diverse array of cell adhesion molecules, toxins, and immune system modulators which are tightly controlled by a large repertoire of regulatory molecules (5).

A basic model of *S. aureus* pathogenesis has virulence progressing in two discrete stages. The first stage is typified by the production of adhesins and surface proteins during the exponential phase of growth. The second stage of infection, analogous to the post-exponential and stationary phases of growth *in vitro*, is characterized by increased toxin production, which leads to increased tissue damage and bacterial spread. The regulatory events controlling this phenotypic switch center on the activities of the *agr* quorum-sensing system, which represses the expression of surface proteins, including protein A (*spa*), and upregulates the production of toxins, such as α -hemolysin (*hla*) and V8 protease (*sspA*), in transition from late exponential to post-exponential phases (22).

The *agr* system is comprised of two divergent transcripts, RNAII and RNAIII. RNAII carries four genes, *agrDBCA*, which are dedicated to the synthesis, sensing, and processing of the quorum-sensing autoinducing peptide (AIP) required for the activation of the RNAII promoter, primarily during the late exponential phase. The divergent transcript RNAIII, activated in response to RNAII via AgrA, is a regulatory RNA molecule expressed maximally during the post-exponential phase and controls virulence gene expression at the transcriptional and translational levels, the latter via an antisense mechanism (11). A number of regulators controlling *agr* expression

(SarA, MgrA, ArlRS, SrrAB, SarX, and SarR) have been described, some with positive effects (e.g., SarA and MgrA) and others with negative effects (e.g., SrrAB and SarX) (7, 10, 15, 19, 20, 27).

Lying adjacent to *sarR*, which encodes a 13.6-kDa protein belonging to the SarA family of DNA binding global regulators in the *S. aureus* chromosome (6), is a gene we called *rsr* (for repressor of *sarR*) that is associated with *sarR* expression. We also confirmed that *rsr* is a repressor of the *agr* system. Additionally, an *rsr* mutant is hypervirulent in a murine model of skin infection. These data suggest that the *rsr* locus is a general inhibitor of virulence in *S. aureus*.

MATERIALS AND METHODS

Reagents, bacterial strains, and growth conditions. Unless specified otherwise, all reagents were obtained from either Fisher (Pittsburgh, PA) or Sigma (St. Louis, MO). The bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains were routinely grown on Luria-Bertani (LB) broth or agar containing 100 μ g/ml of ampicillin where appropriate. *S. aureus* cultures were routinely cultured on either tryptic soy broth or agar. Strains carrying plasmids were selected and maintained on either 2.5 μ g/ml erythromycin or 10 μ g/ml chloramphenicol, as appropriate. Experiments investigating *agr* transcription were carried out in 03GL broth (23).

Cultures for gene and protein expression analysis were grown in either glass sidearm flasks or 18-mm glass tubes in an Excella E24 shaking incubator set at 37°C and 250 rpm (New Brunswick Scientific, Edison, NJ). Growth was monitored by determining culture turbidity at 650 nm with an 18-mm borosilicate glass tube using a Spectronic 20D+ spectrophotometer (Spectronic Analytical Instruments, Garforth, England). Data collected at mid-, late, or post-exponential phases of growth corresponded to turbidity readings of 0.7, 1.1, and 1.7, respectively.

Genetic manipulations. A list of oligonucleotides used in this study is available from the authors. Enzymes and reagents used for genetic manipulations were obtained from either Invitrogen (Irving, CA) or New England Biolabs (Ipswich, MA) and used according to the manufacturers' recommendations. Plasmids were originally propagated in *E. coli* and transformed by electroporation into RN4220, a heavily mutagenized, restriction-deficient strain of *S. aureus* capable of methylating foreign DNA. The methylated plasmid DNA was then electroporated into the appropriate *S. aureus* strains. All positive clones were initially identified by PCR and confirmed by DNA sequencing.

A markerless, in-frame deletion of *rsr* was constructed in four *S. aureus* strains using a PCR-based splicing technique followed by splicing overlap extension (SOEing), as described previously (2, 26). Briefly, the fragment was cloned into pMAD and introduced into *S. aureus* strains SH1000, Newman, RN6390, and

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
<i>S. aureus</i>		
RN4220	Heavily mutagenized strain that accepts foreign DNA, <i>agr</i> mutant	23
SH1000	8325-4 with <i>rsbU</i> restored	14
COL	A methicillin-resistant strain, isolated from a human infection in the early 1960s	13
Newman	Methicillin-sensitive laboratory strain, isolated from a human infection in 1952	3
RN6390	Laboratory strain related to strain 8325-4	25
ALC4657	SH1000 Δ <i>rsr</i>	This work
ALC4658	COL Δ <i>rsr</i>	This work
ALC4659	Newman Δ <i>rsr</i>	This work
ALC4660	RN6390 Δ <i>rsr</i>	This work
ALC6582	SH1000 Δ <i>rsr/rsr</i> complement	This work
ALC6583	SH1000 Δ <i>sarR</i>	This work
ALC6584	SH1000 Δ <i>sarR</i> Δ <i>rsr</i>	This work
ALC2386	SH1000 <i>agr::tetM</i>	14
ALC6585	SH1000 <i>agr::tetM</i> Δ <i>rsr</i>	This work
ALC6586	SH1000 with pEPSA5:: <i>rsr</i>	This work
<i>E. coli</i>		
TOP10	General cloning strain	Invitrogen
Plasmids		
pMAD	<i>E. coli-S. aureus</i> shuttle vector containing a thermosensitive origin of replication <i>bgab</i> , Em ^r , Ap ^r	2
pEPSA5	<i>E. coli-S. aureus</i> shuttle vector containing a xylose-inducible promoter, Ap ^r , Cm ^r	9
pALC1484	<i>E. coli-S. aureus</i> shuttle vector derived from pSK236 containing a multiple cloning site upstream of the <i>gfp_{uvr}</i> gene (a <i>gfp</i> variant optimized for expression in <i>S. aureus</i>), Ap ^r , Cm ^r	16
pALC1743	<i>agr</i> P3 promoter driving the expression of <i>gfp_{uvr}</i> in pALC1484	16
pALC1741	<i>spa</i> promoter driving the expression of <i>gfp_{uvr}</i> in pALC1484	17
pALC1740	<i>hla</i> promoter driving the expression of <i>gfp_{uvr}</i> in pALC1484	17
pALC2831	<i>sspA</i> promoter driving the expression of <i>gfp_{uvr}</i> in pALC1484	26

COL (to obtain *rsr*) or ALC2386 (to obtain the *rsr-agr* mutant in SH1000) by electroporation, followed by the selection of blue colonies. Transformants were grown at 42°C, a nonpermissive temperature for pMAD replication, and then at 30°C to drive homologous recombination to yield white colonies (2, 26). A similar approach was used to construct the *rsr-sarR* double mutant in the SH1000 background, using primers flanking both *sarR* and *rsr*.

The mutation in the *rsr* mutant was restored by cloning the wild-type gene into pMAD and reintroducing it into the chromosome by homologous recombination, as described above. The *rsr*-overproducing strain was created by cloning the *rsr* open reading frame behind the xylose-inducible promoter of the plasmid pEPSA5 (9).

Transcriptional analysis by Northern blotting. Cells from either the mid-, late, or post-exponential phases of growth were harvested, resuspended in Trizol (Invitrogen), and lysed using 0.1-mm silica-zirconia beads in a reciprocating shaker. The RNA was extracted and resuspended in diethyl pyrocarbonate (DEPC)-treated water, according to the manufacturer's instructions. Ten micrograms of RNA was separated electrophoretically on 1.5% agarose-0.66 M formaldehyde gels in MOPS buffer (20 mM morpholinepropanesulfonic acid, 10 mM sodium acetate, 2 mM EDTA [pH 7]), transferred onto a Hybond XL nylon membrane (GE Healthcare) in 20× SSC (3 M NaCl, 0.3 M sodium citrate [pH 7]), and fixed to the membrane by cross-linking in a Stratilinker 1800 (Stratagene, La Jolla, CA).

The DNA probes, generated by either PCR or restriction digestion, were labeled with [α -³²P]dCTP using the Roche random prime labeling kit (Hoffmann-La Roche Inc., Nutley, NJ) and added to the nylon membranes at 65°C. After hybridization overnight, the membranes were washed extensively and autoradiographed. Blots shown in Fig. 1, 2, 3, and 6 are representative of results from three separate experiments, and the intensities of the resulting bands were quantified using ImageJ (1).

Real-time PCR analysis of gene transcription. RNA, prepared as described above, was treated with RNase-free DNase to remove residual DNA, according to the manufacturer's instructions (Turbo-DNAfree; Applied Biosystems/Ambion, Austin, TX). Successful removal of the DNA was confirmed by PCR analysis. RNA (1 μ g) was reverse transcribed using the Roche Transcriptor first-strand cDNA synthesis kit, and the resulting cDNA levels were quantified using the Maxima SYBR kit from Fermentas (Glen Burnie, MD) in a Roche

LightCycler 1.5 according to the manufacturer's protocols. Reported levels of gene expression were normalized to those of *gyrB*.

Detection of SarR and Rsr by Western blotting. Post-exponential-phase (optical density at 650 nm [OD₆₅₀] of 1.7) or late-exponential-phase (OD₆₅₀ of 1.1) cells were collected and lysed in 10 mM Tris, 1 mM EDTA (pH 8) with 0.1-mm silica-zirconia beads in a reciprocating shaker. Soluble cellular proteins were separated from debris by a high-speed spin at 12,000 × g for 5 min at 4°C and quantified using the BCA protein assay kit (Fisher). Equal amounts (20 or 75 μ g) of protein were then separated on a 12.5% polyacrylamide-SDS gel and transferred onto a polyvinylidene difluoride membrane. The presence of SarR or Rsr was detected on the membranes using standard Western blotting procedures. The primary antibody was a murine-derived anti-SarR monoclonal antibody (clone 1D1) or murine anti-Rsr polyclonal antibody used at a concentration of 1:1,000, and the secondary antibody was donkey-derived anti-mouse immunoglobulin G conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) and used at a concentration of 1:10,000. After a series of extensive washes, an enhanced chemiluminescence kit (GE Healthcare) was used to detect binding of the antibodies to the membrane. Where applicable, ImageJ was used to quantify the relative intensities of the resulting bands. Results shown are representative of three separate experiments.

Transcriptional analysis using green fluorescent protein (GFP)-promoter fusions. Shuttle plasmids containing the promoter regions of various genes (Table 1) driving the expression of GFP_{uvr} (16) were transformed into SH1000, the *rsr* mutant, and the complemented mutant by electroporation. The resulting strains were grown overnight in tryptic soy broth (TSB), diluted 1:100 in fresh TSB the next morning, and incubated at 37°C with shaking at 250 rpm. Growth (OD₆₀₀) and fluorescence (excitation/emission of 485/515 nm) of the cultures were monitored with an FL600 microplate fluorescence reader (BioTek Instruments, Winooski, VT). Experiments were performed three times using four independently isolated clones in triplicate. Error bars represent the standard errors of the means, and statistical significance was determined by the Student *t* test.

Subcutaneous murine model of skin infection. SH1000 and its isogenic *rsr* mutant were grown in TSB to post-exponential phase (OD₆₅₀ of 1.7), harvested, washed three times in sterile saline, and resuspended in sterile saline at 10⁹ CFU/ml. CFU (10⁸) in 100 μ l of SH1000 or the *rsr* mutant were injected subcutaneously into the shaved backs of six 12-week-old male C57BL/6 mice in the

SH1000 group or in the *rsr* mutant group (Jackson Labs, Bar Harbor, ME). One mouse from the SH1000 group died upon handling. The mice were then monitored daily for subcutaneous abscess formation and other changes in general appearance (e.g., ruffled hair and activity). On the 8th day postinfection, the mice were sacrificed and 8-mm punch biopsy specimens of the skin were obtained from the infected sites. A portion of the skin biopsy specimen was fixed in 10% neutral buffered formalin for histological analysis; the remainder was processed in a glass tissue grinder and serially diluted to determine bacterial counts. Results reported are the means relative to the mass of the tissue section in grams. Statistical significance was determined using the Student *t* test.

Samples for histology were embedded in paraffin, sectioned at 5 μ m, and stained with hematoxylin and eosin according to standard histological procedures. Slides were viewed and images were captured using an Olympus digital light microscope (Olympus America Inc., Center Valley, PA).

RESULTS

Identification of *rsr*. Recent complementation studies of the *sarR* mutant from our lab suggested that the genetic locus upstream of *sarR* may inhibit *sarR* expression. We have termed this locus *rsr*, for repressor of *sarR*. *rsr* is a unique gene bearing no homology to any gene outside the staphylococci, nor does it have any conserved motifs or domains. It is predicted to encode a 108-amino-acid protein with a molecular mass of \sim 12.5 kDa and a theoretical pI of 4.54.

The *rsr* locus neighbors other regulatory genes (Fig. 1A). Besides being upstream of *sarR*, *rsr* is also directly downstream of *sarY*, another member of the SarA protein family (5, 6). An uncharacterized gene, SA2092, lies upstream of *sarY* and is homologous to the AraC family of regulators. Despite its proximity to these regulatory genes, *rsr* appeared to be transcribed monocistronically with a transcript size of \sim 400 nucleotides (nt), corresponding to the size of the *rsr* gene plus the intergenic region at the 5' end (Fig. 1A and B). Analysis of *rsr* expression during different phases of *S. aureus* growth revealed that the gene was most highly transcribed during mid-exponential phase; however, the levels of the Rsr protein produced appeared to be stable throughout the growth period (Fig. 1B).

To investigate the phenotypic and genotypic effects of *rsr*, we first constructed an in-frame deletion of *rsr* in *S. aureus* strain SH1000, chosen for its similarity to the commonly published strains, including 8325-4 and RN6390, and its possession of an intact *rsbU* gene within the stress-induced *sigB* operon. As expected, the *rsr* mutant of SH1000 did not express the cognate transcript, while restoration of a single copy of *rsr* to the mutant chromosome reestablished the transcription of *rsr* (Fig. 1C). Immunoblot analysis demonstrated that the Rsr protein was absent in the *rsr* mutant but was expressed in the parent SH1000 strain and the restored *rsr* mutant strain (Fig. 1D).

Repression of SarR by *rsr*. As described above, we initially suspected that *rsr*, a gene adjacent to *sarR*, may be a repressor of SarR expression in *S. aureus*. To verify the impact of *rsr* on *sarR* expression in a variety of prototypic *S. aureus* strains, including SH1000, we also deleted *rsr* in RN6390, Newman, and COL, thus encompassing both methicillin-susceptible (MSSA) and methicillin-resistant *S. aureus* (MRSA) strains. Lysates of *rsr* mutants from strains SH1000, RN6390, Newman, and COL prepared from post-exponential-phase cells (corresponding to the peak expression period for *sarR*) were immunoblotted and probed with an anti-SarR monoclonal antibody. In all four genetic backgrounds, expression of the SarR protein was increased in the mutant compared to that in the cognate

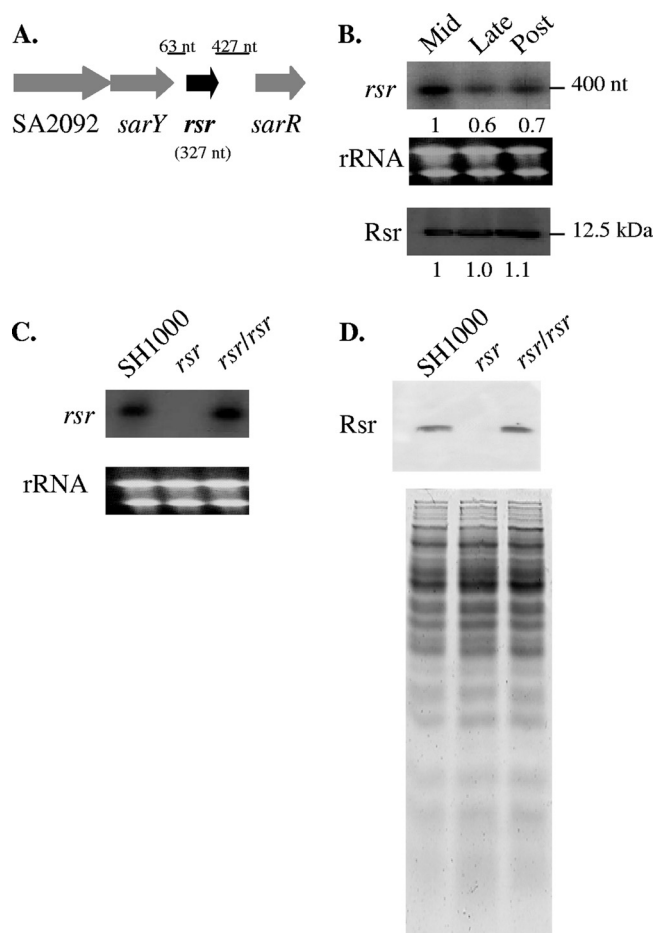


FIG. 1. (A) Genomic context of the *rsr* gene. *rsr* lies in close proximity to three regulatory genes, *sarR*, *sarY*, and SA2092, which encodes a gene product with similarity to the AraC protein family. (B) Transcription (top) of *rsr* during the early, mid-, and late exponential phases of growth in strain SH1000. Numbers underneath the panels indicate relative intensities of the bands as determined by ImageJ. rRNA bands (middle) are shown as a loading control for the top panel. (C) Transcription (top) of *rsr* during the mid-exponential phase in the SH1000 parent, *rsr* mutant, and restored *rsr* mutant with a single copy of the *rsr* gene at its native chromosomal location. The intensities of the rRNA bands (bottom) serve as loading controls. (D) Translation of Rsr in the parent, mutant, and restored *rsr* mutant strains at late exponential phase as detected by a Western blot, with 20 μ g of whole-cell lysate each and probed with a 1:1,000 dilution of murine anti-Rsr antibody (top). The bottom panel represents an equivalent SDS gel stained with Coomassie blue to demonstrate comparable loading among the lanes.

parent, ranging from an \sim 20% increase in the Newman mutant to \sim 60% in the RN6390 mutant compared with the corresponding parent, thus demonstrating the repressive effect of *rsr* on SarR in a variety of strains (Fig. 2A). Based on these trends, we decided to focus on SH1000, predicated upon the wealth of information we and others have generated on the regulatory pathway of *agr* and *sarA* in this strain (15, 20, 21, 29). Using the *rsr* mutant and the restored *rsr* mutant in which the mutated gene was replaced by the wild-type copy, we analyzed *sarR* transcription and showed that the *sarR* transcript level was increased in the *rsr* mutant compared with that in the cognate parent SH1000 strain but returned to the parental

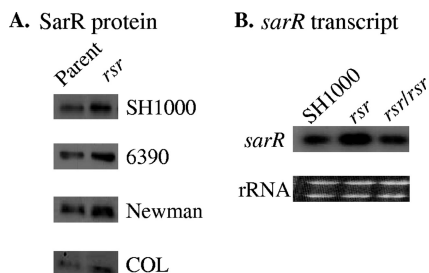


FIG. 2. Effect of an *rsr* mutation on the translation (A) and transcription (B) of *sarR*. (A) Western blot analysis of SarR expression in *rsr* mutations in four *S. aureus* strains. Cell lysates were prepared from late-exponential-phase cells and probed with a murine anti-SarR monoclonal antibody. Expression of the SarR protein in the *rsr* mutants was 1.4, 1.6, 1.2, and 1.5 times that of the respective parents in SH1000, RN6390, Newman, and COL, as determined by ImageJ. (B) Northern blot analysis of *sarR* transcription during the late exponential growth phase in the SH1000 parent, the *rsr* mutant (expression at 1.7 times that of SH1000), and the restored *rsr* mutant. rRNA bands are shown as loading controls.

level in the restored *rsr* mutant, strongly suggesting that *rsr* was repressing *sarR* expression (Fig. 2B).

Role of *rsr* in virulence gene transcription. Given the role of *agr* and *sarA* in modulating expression of many virulence genes (19, 21), we next examined the transcript levels of these two regulatory loci in the *rsr* mutant compared to those in the parent SH1000 strain and the restored *rsr* mutant strain. Curiously, the levels of *sarA* transcription were virtually identical among all 3 strains (data not shown), thus implying that *rsr* does not play a major role in controlling *sarA* expression. We then assessed *agr* expression by ascertaining the levels of RNAII (carrying the machinery of the *agr* system, *agrDBCA*),

normally expressed maximally during the late exponential phase (OD₆₅₀ of 1.1). As shown in Fig. 3A, the transcription of RNAII was significantly increased in the *rsr* mutant compared to that in the parent and the restored *rsr* mutant. As RNAII augments RNAIII (the *agr* effector regulatory molecule), we also found that RNAIII expression was elevated in the *rsr* mutant compared to that in the cognate parent and restored mutant (Fig. 3A). GFP fusion analysis of the *agr* RNAIII promoter confirmed the Northern blot data (Fig. 3B). We also examined the expression of virulence genes controlled by *agr*. More specifically, the transcription of *hla* (encoding α -hemolysin), *sspA* (encoding V8 protease), and *spa* (encoding protein A) was also affected upon deletion of *rsr*, with both *hla* and *sspA* transcription upregulated by 2- to 3-fold in the *rsr* mutant compared to that in the parent and the restored *rsr* mutant, as assessed by Northern blot and promoter fusion analyses (Fig. 3A, C, and D). In contrast, the transcription of *spa* was downregulated in the *rsr* mutant compared to that in the parent and the restored *rsr* mutant (Fig. 3A and E). The upregulation of *hla* and *sspA* and the downregulation of *spa* in the *rsr* mutant of SH1000 is in concordance with upregulation of RNAII and RNAIII in this strain.

Ascertaining the linkage between *rsr* and *agr*. Based on the above transcriptional data, we wanted to determine if *rsr* requires SarR to impact *agr* and the ensuing downstream genes, such as *hla*, *sspA*, and *spa*. For this purpose, we first constructed an in-frame deletion mutant of both *sarR* and *rsr* to determine the role of *sarR* in mediating the effect of *rsr* on *agr*. Cognizant of the fact that the *agr* effector molecule RNAIII is maximally expressed during the post-exponential phase (OD₆₅₀ of 1.7), we used real-time PCR transcription of RNAIII in the *rsr-sarR* double mutant during this growth

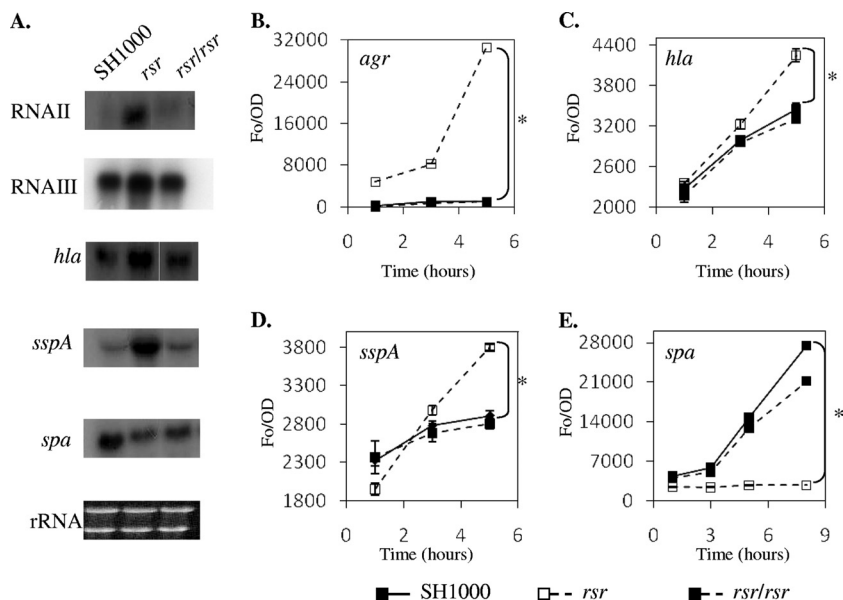


FIG. 3. Effect of an *rsr* mutation on *agr* RNAII and RNAIII, *hla*, *sspA*, and *spa* transcript levels (A) and promoter activities (B to E). (A) Northern blot analysis of RNA isolated from postexponential-phase (RNAII, RNAIII, *hla*, and *sspA*) or late-exponential-phase (*spa*) cells. Late exponential and postexponential phases are defined as an OD₆₅₀ of 1.1 and 1.7, respectively, using 18-mm borosilicate glass tubes in a Spectronic 20D+ spectrophotometer. (B to E) GFP-promoter fusion analysis of the *agr*P3, *hla*, *sspA*, and *spa* promoter activities, respectively, over the course of bacterial growth in the SH1000, *rsr* mutant, and restored *rsr* mutant strains. *, *P* < 0.01, as determined by the Student *t* test.

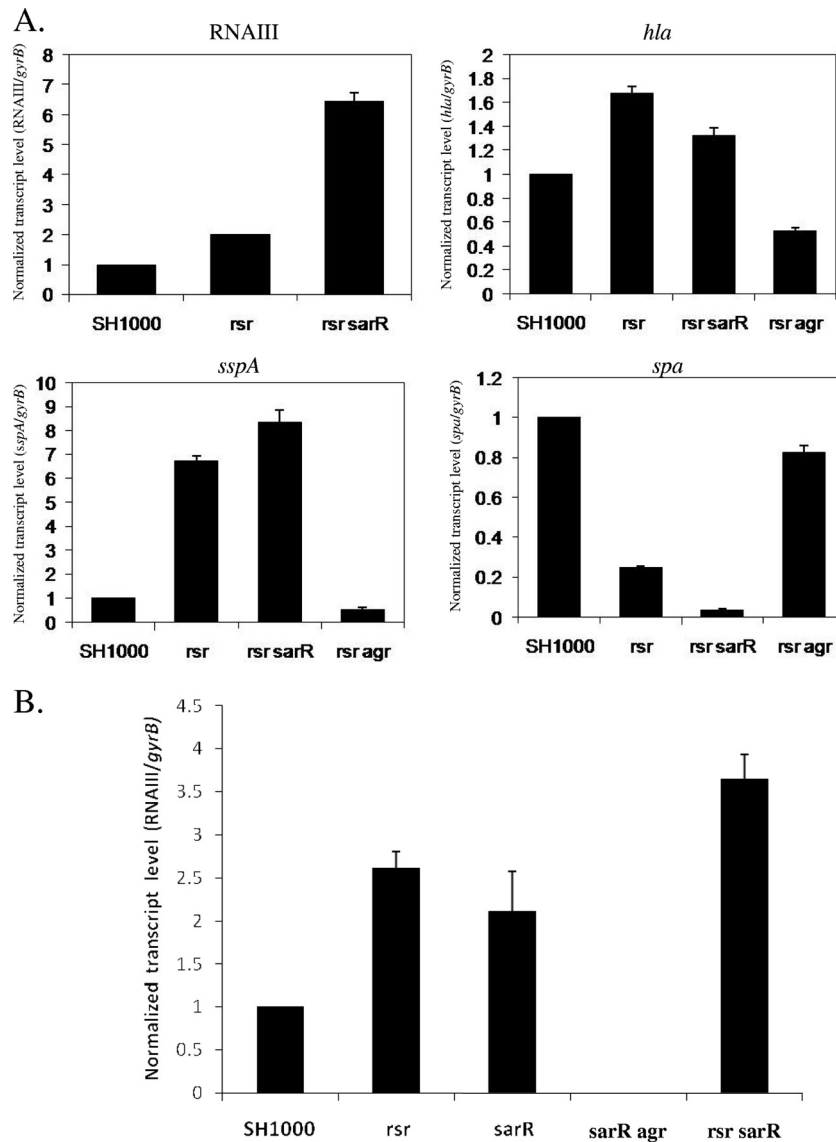


FIG. 4. (A) Transcriptional analysis of RNAIII, *hla*, *sspA*, and *spa* in an *rsr-sarR* and an *rsr-agr* double mutant from post-exponential-phase cells (OD₆₅₀ of 1.7), treated with RNase-free DNase, reverse transcribed into cDNA, and analyzed by real-time PCR. (B) Real time-PCR of RNAIII of late-exponential-phase cells (OD₆₅₀ of 1.1) from *rsr*, *sarR*, *sarR-agr*, and *rsr-sarR* mutants. Transcription levels in the mutants are normalized to the levels of *gyrB* and are reported relative to their transcription in the SH1000 parent (set to 1). The error bars represent individual standard deviations. The *P* values for SH1000 compared to the single *rsr* mutant, *sarR* mutants, and double mutants are <0.0008, <0.02, and <0.0006, respectively. The *P* values for the *rsr-sarR* double mutant compared to the *rsr* single mutant and the *sarR* mutant are <0.005 and <0.008, respectively.

phase. As shown in Fig. 4A (top left), the transcription of RNAIII was more pronounced in the double *rsr-sarR* mutant than in the *rsr* single mutant by a factor of 3.5 and in the parent SH1000 by a factor of 6.7. As expected, the transcription of *hla* in the *rsr* and *rsr-sarR* mutants was increased compared with that in the parental SH1000 strain. However, *hla* expression in the *rsr* mutant was 1.3-fold higher than in the double *rsr-sarR* mutant (Fig. 4A, top right). With respect to *sspA* and *spa* transcription, the *rsr-sarR* double mutant demonstrated a phenotype consistent with a higher level of RNAIII than the single *rsr* mutant, with *sspA* transcript levels increased by 1.3-fold and *spa* transcription significantly decreased in the double mutant compared to that in the *rsr* single mutant (Fig. 4A, bottom).

We also analyzed the effect of *rsr* on RNAIII during the late exponential phase (OD₆₅₀ of 1.1). Similar to the findings during the post-exponential phase, the double *rsr-sarR* mutant exhibited a higher level of RNAIII expression than either the single *rsr* or the *sarR* mutant, as assessed by real-time PCR (Fig. 4B). More importantly, both *rsr* and *sarR* mutants demonstrated higher RNAIII expression than the parental SH1000 strain. Based on the above data with RNAIII, *sspA*, and *spa*, our results suggested that *agr* and its downstream effectors are repressed by *rsr*. This repressive effect of *rsr* is likely independent of *sarR*, because RNAIII expression is higher in the *rsr-sarR* double mutant than in the single *rsr* and *sarR* mutants.

To determine if the virulence genes controlled by *rsr* are

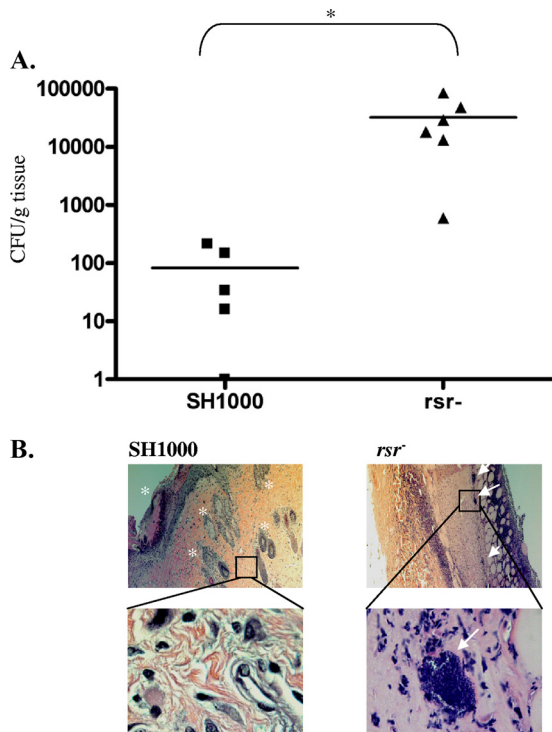


FIG. 5. Virulence of the *rsr* mutant. (A) A group of six C57BL/6 mice were each injected subcutaneously with 10^8 CFU of parent SH1000 or the *rsr* mutant and monitored daily. Eight days postinfection, the mice were sacrificed, and punch biopsies of the skin were done to determine bacterial load (*, $P < 0.01$, as determined by the Student *t* test) and to examine any histological differences between the mice from the parental SH1000 group (left) and the *rsr* mutant group (right). (B) Top, low magnification ($100\times$) of the dermal and subdermal layers. Note the absence of the dermal layer and subcutaneous structures (*) in the biopsy specimen taken from a mouse infected with the *rsr* mutant and the presence of clusters of bacteria (arrows). Bottom, higher magnification ($1,000\times$) of a representative bacterial cluster (right, arrow) present in the *rsr* mutant-infected tissue (right) and an equivalent region from the SH1000-infected tissue (left).

totally dependent on *agr* repression, we constructed an *rsr-agr* double mutant, followed by transcriptional analysis of virulence genes in this strain by real-time PCR. In contrast to the *rsr-sarR* double mutant, the transcriptional patterns of the *rsr-agr* double mutant differed significantly from that of the single *rsr* mutant, with both *hla* and *sspA* transcription markedly reduced (over 50% reduction) compared to that in the parental strain, while *spa* transcription was almost fully restored to the parental level (Fig. 4A), again reaffirming the notion that the regulation of these effector genes by *rsr* is mediated via RNAPIII of the *agr* locus (Fig. 4A).

Hypervirulence of an *rsr* mutant. Based on the transcriptional profile of increased *agr* transcription in the *rsr* mutant, we reasoned that the mutant might be hypervirulent compared with the parent. To test this hypothesis, we employed a murine model of cutaneous infection. For this model, we injected 10^8 CFU/100 μ l of SH1000 or the *rsr* mutant subcutaneously into the shaved backs of 6- to 12-week-old male C57BL/6 mice. On the first day postinfection, all of the mice in the mutant group had developed small areas of redness near the injection site, whereas none of the mice in the

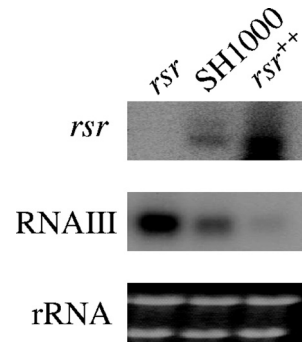


FIG. 6. *rsr* as an inhibitor of virulence gene regulators. The *rsr* gene was overexpressed in SH1000 using a multicopy plasmid pEPSA5 containing *rsr*, and the transcript levels of *rsr* and *agr* RNAPIII were assessed by Northern blotting using RNA extracted from post-exponential-phase cells (OD_{650} of 1.7). The expression of *rsr* and RNAPIII in the *rsr* mutant expressing a high copy number of *rsr* was 4 times and 0.2 times that of the parental SH1000 strain, using ImageJ to determine the relative band intensities.

parental SH1000 group did. As the infection progressed, the mice infected with SH1000 did eventually develop small, red lesions on their backs that were indistinguishable from those of the mice in the *rsr* mutant group. The bacterial loads recovered from the tissue biopsy specimens were markedly different between the two groups of mice. On average, 83 and 32,500 CFU/gram of tissues were recovered from the mice in the SH1000 and *rsr* groups, respectively (Fig. 5A). The histological profile of the tissue sections taken from the mice reflected their bacterial burden. Skin tissue samples taken from the mice infected with SH1000 had normal skin architecture with increased infiltration of phagocytic cells in the dermis (Fig. 5B, left) and a lack of tissue necrosis. Importantly, few, if any, bacteria were present in the infected tissue. Mice infected with the *rsr* mutant, in contrast, revealed abnormal skin histology; in particular, there was a massive infiltration of neutrophils and monocytes to the dermal and subdermal layers (Fig. 5B, right). The epidermal layer was absent, and several subcutaneous structures, such as hair shafts and sebaceous glands, were obliterated. Additionally, large clusters of bacteria were visible in the deep muscle tissue (Fig. 5B, bottom right).

***rsr* as an inhibitor of *agr*.** Both transcriptional analysis of virulence genes and the results obtained from the murine model of cutaneous infection suggested that the *rsr* mutant possessed an increased virulence phenotype. We thus considered the possibility that provision of *rsr* in multiple copies to *S. aureus* may decrease its virulence properties. To test this hypothesis, *rsr* was cloned downstream of the xylose-inducible promoter of the pEPSA5 plasmid and transformed into strain SH1000. The levels of *rsr* and RNAPIII transcription were then assessed in the resultant strain, the *rsr*⁺⁺ strain. As shown in Fig. 6, the level of *rsr* transcription was approximately 4-fold higher in *rsr*⁺⁺ than in SH1000. The increased gene dosage of *rsr* in the *rsr*⁺⁺ strain resulted in a dramatic reduction (~80%) in *agr* RNAPIII transcription compared with that in SH1000.

DISCUSSION

In this study, we report the identification of *rsr*, a gene unique to the staphylococci that bears no similarity to any known gene or conserved domain. Inactivation of *rsr*, residing in a chromosomal location directly adjacent to *sarR*, resulted in overproduction of the virulence gene regulator SarR in four *S. aureus* strains. Using SH1000 as the genetic backdrop, we found that the *rsr* mutant also exhibited augmented *agr* (RNAII and RNAIII), *hla* (α -hemolysin), and *sspA* (V8 protease) transcription as well as a decrease in the *spa* (protein A) transcript level, consistent with an increase in *agr* expression in the *rsr* mutant. As SarA is a positive regulator of *agr*, we also analyzed *sarA* transcription in the *rsr* mutant of *S. aureus* strain SH1000 and found comparable levels of *sarA* transcription among the mutant, parent, and *rsr*-restored mutant strains.

The *rsr* gene was transcribed most highly during the mid-exponential phase of growth; however, analysis of Rsr suggested that the protein level was relatively constant throughout the growth cycle. This observation suggested that the Rsr protein may not be involved in the temporal control of gene expression unlike several other *S. aureus* regulatory loci but may act throughout the growth cycle (4, 5). Indeed, changes in activity of both the *agr* and *spa* promoters, which exhibited the most striking changes in the *rsr* mutant, were sustained over the entire growth cycle (Fig. 3B and D). On the other hand, the promoter activities of *hla* and *sspA*, which are most active after the exponential phase, remained subdued in the mutant until the cells reached post-exponential phase (Fig. 3C and D), at which time these promoters became much more active than those in the parent, suggesting that regulatory factors other than *rsr* may have been at play in controlling these genes.

Although *rsr* is a repressor of *sarR* (Fig. 2) and *agr* (Fig. 3), we found that increased *agr* RNAIII expression in the *rsr* mutant is not attributable to enhanced *sarR* expression, because RNAIII expression in the double *rsr-sarR* mutant was higher than that in the single *rsr* and *sarR* mutants (Fig. 4B). More specifically, analysis of *agr* expression in the *rsr-sarR* double mutant indicated that the derepressive effects of *rsr* and *sarR* mutations on *agr* RNAII and subsequently on RNAIII appear to be additive (e.g., compare the RNAIII expression in *rsr*, *sarR*, and double *rsr-sarR* mutants in Fig. 4B). Consistent with this result was the observation that the effects of concomitant *rsr* and *sarR* mutations in SH1000 on *sspA* and *spa* expression also appeared to be culminative (Fig. 4A). These results indicated that either *rsr* directly represses *agr* transcription or does so via another regulatory molecule which acts as an intermediary between *rsr* and *agr*. In addition, the effect of *rsr* on *agr* expression is independent of SarR. The finding that the introduction of an *agr* mutation in an *rsr* mutant can downregulate *hla* and *sspA* as well as upregulate *spa* expression to near parental levels (Fig. 4) could lend support to the notion that *rsr* regulates virulence determinants primarily via an intact *agr* pathway (Fig. 7).

The transcriptional changes observed in the *rsr* mutant implied that its virulence phenotype may also have been enhanced. These phenotypic changes, however, were difficult to assess in SH1000 because this strain, by virtue of the restored *rsbU* gene, displays an enhanced *sigB* response and hence a more muted *agr* response (14), resulting in very low levels of

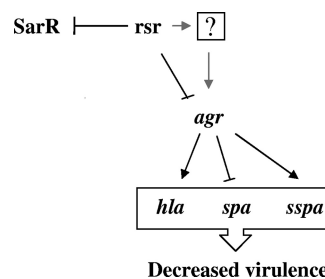


FIG. 7. Proposed mechanism of *rsr*-mediated gene regulation. *rsr* represses both *sarR* and *agr* during the late exponential phase. The repressive effects on *agr* by *rsr* during the late exponential phase are shown with regard to *hla*, *sspA*, and *spa* transcription. It is unclear whether *rsr* represses *agr* directly or via an as-of-yet-unidentified regulator. The repression of *agr* contributes to the repression of both *hla* and *sspA* and to the activation of *spa*. Together, these regulatory changes are predicted to result in a net decrease in virulence. The genetic locus *rsr* thus acts independently of SarR to inhibit *agr* expression.

hemolysis and proteolysis activities *in vitro*. We therefore decided to evaluate the clinical significance of *rsr* *in vivo* by comparing the virulence properties of the parent and mutant strains in a murine model of cutaneous infection. As expected, SH1000 was not proficient in causing subcutaneous abscesses in the mouse model, requiring up to 10^8 CFU to establish a mild abscess in the parent group. With the exception of a few erythematous lesions, most of the mice seemed unaffected initially by the subcutaneous injection of 10^8 CFU of strain SH1000. Histological studies also disclosed the integrity of the epidermis and dermis layers of the skin, thus corroborating with the clinical observation of these mice, which appeared relatively healthy. In contrast, subcutaneous injection of an equivalent dose of the *rsr* mutant offered dramatically different results, with on average almost 100-fold more bacteria recovered from the injection sites of the *rsr* group than from those of the parental group. Histological findings of mice infected with the *rsr* mutant also bore several classical hallmarks of infection/inflammation, with disruption of the epidermis accompanied by massive neutrophilic infiltration of the dermis layer.

Accordingly, this work extends our knowledge of the regulatory cascade controlling virulence in *S. aureus*. Our studies have identified *rsr* as the first known regulator of *sarR*. Besides repressing *sarR* expression, *rsr* also downregulates *agr* transcription. This reduction in *agr* expression was not due to changes in *sarA* expression. In addition, both *rsr* and SarR appear to repress *agr* expression in an additive manner during the late exponential phase, as exemplified by enhanced RNAII and RNAIII expression in the double *rsr-sarR* mutant over the single *rsr* and *sarR* mutants (Fig. 4). Whether the *rsr*-mediated effect on *agr* during the late exponential phase is direct or indirect via another regulator remains to be defined. However, we cannot directly infer from our studies here the role of *rsr* on stationary-phase expression of virulence determinants, because we have found that heightened expression of RNAIII during the stationary phase is less dependent on *rsr* (unpublished data). Nevertheless, it is clear that a mutation in *rsr* has led to an enhanced virulence response in our murine cutaneous model. Indeed, overexpression of *rsr* in the parental SH1000 strain, as expected, decreased the transcription of RNAIII of

agr, and consequently, one would predict a corresponding decrease in virulence effectors and hence disease progression if *rsr* is upregulated *in vivo*.

One relevant question regarding our findings is the plausible mechanism by which a unique molecule like *rsr*, which is present only in staphylococcal species, could inhibit expression of *agr*-controlled virulence determinants. We have found that the Rsr protein does not bind to the *agr* promoter, thus ruling out direct protein-DNA binding as a mode of regulation. Additionally, pulldown studies showed that Rsr and SarR do not interact at the protein-protein level. Interestingly, model analysis of *S. aureus* mRNA structure revealed that the ~400-nt *rsr* mRNA has features consistent with a small antisense RNA (12, 24). This mRNA, which is transcribed most highly during mid-exponential growth, may then regulate the target gene function early during the growth cycle. Work is ongoing to investigate this possibility.

Additional work in our lab is directed at determining whether these regulatory pathways are found in more clinically relevant strains, such as the community-acquired strains USA300 and USA400. If so, developing the means to either increase the levels of *rsr* or augment its activities may lead to the development of novel anti-infectives toward *S. aureus*.

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