# Repression of *Staphylococcus aureus* SrrAB Using Inducible Antisense *srrA* Alters Growth and Virulence Factor Transcript Levels†

Alexa A. Pragman,‡ Yinduo Ji,§ and Patrick M. Schlievert*,‡

*Department of Microbiology, University of Minnesota, Minneapolis, Minnesota 55455, and Department of Veterinary Biosciences, University of Minnesota, St. Paul, Minnesota 55108*

*Received February 15, 2006; Revised Manuscript Received September 24, 2006*

ABSTRACT: Our laboratory has previously identified the staphylococcal respiratory response (SrrAB), a *Staphylococcus aureus* two-component system that acts in the global regulation of virulence factors. In strain RN4220, SrrAB downregulated production of *agr* RNAIII, protein A, and TSST-1, particularly under low-oxygen conditions. Work by another group showed that SrrAB regulates energy metabolism genes and indicated that SrrAB may regulate energy transduction in response to changes in oxygen availability. In this study we investigate the role of SrrAB in regulating RNAIII, *tst*, *spa*, *icaR*, and *icaA* in a clinical isolate of *S. aureus*, MN8. We employ an inducible antisense vector, pYJY4, in order to repress transcription of *srrAB*. Transcript levels were assessed by reverse transcription followed by quantitative PCR. Repression of *srrAB* in rich media under aerobic growth conditions shows that SrrAB is required for expression of *tst*, *spa*, and *icaR* transcripts at wild-type levels. Comparisons made between rich media under aerobic conditions vs low-oxygen conditions show that *srrAB* transcript levels are not altered by oxygen alone. Previous studies performed on strain RN4220 under low-oxygen conditions indicate that SrrAB represses *tst* and *spa* transcript when the amount of oxygen is limited. We propose that, under aerobic conditions, SrrAB enhances the levels of *tst*, *spa*, and *icaR*, while under low-oxygen conditions, SrrAB decreases the levels of these three transcripts.

*Staphylococcus aureus* is a Gram-positive pathogenic microorganism that causes a variety of illnesses in humans and animals. *S. aureus* infections can range from simple skin abscesses or boils to severe, life-threatening infections such as endocarditis, osteomyelitis, necrotizing pneumonia, or toxic shock syndrome (TSS) (*1*). The ability of the organism to cause severe disease is thought to be due to the coordinated expression of virulence factors that is mediated by staphylococcal virulence regulators.

Many global regulators of virulence have been described in *S. aureus*, both in vitro and in vivo (*2*). Of these regulators, the accessory gene regulator system (*agr*)[1] is the most well-studied regulator of virulence factors. The *agr* locus consists of a quorum-sensing two-component system and a divergently transcribed regulatory RNA molecule, which is the effector molecule of the *agr* system. The two divergently transcribed promoters direct the transcription of RNAII, which encodes the AgrBDCA proteins, and RNAIII, the effector RNA (*3*, *4*). Consistent with its function as a quorum-sensing system, RNAIII expression is highest in postexponential and stationary growth phases in vitro. Expression of RNAIII results in an increase in exotoxin expression and a decrease in the expression of surface-associated virulence factors. Although in vitro studies show *agr* to be a cell density-dependent global regulator of virulence factors, several in vivo experiments demonstrate that RNAIII is repressed in animal infection models (*5*, *6*) as well as during human lung infection (*7*). Recent work on *agr* has shown that *agr* is expressed by *S. aureus* inside the host cell endosome and appears to play a role in internalization of the bacteria and induction of host cell apoptosis (*8*, *9*).

The staphylococcal accessory gene regulator (*sarA*) and the staphylococcal accessory element (*saeRS*) are two other global regulators of virulence in *S. aureus*. *SarA* is a DNA-binding transcription factor that activates transcription of extracellular proteins (α- and *β*-hemolysins, toxic shock syndrome toxin 1, and staphylococcal enterotoxin B) and cell-surface proteins such as fibronectin binding protein while repressing protease activity (*10*). In addition, SarA activates transcription of both *agr* promoters (*11*). SarA acts through the *agr* system to mediate its effects as well as acting independently of *agr* (*11*−*15*). The *saeRS* locus encodes a two-component system that regulates α- and *β*-hemolysin and coagulase at the level of transcription. SaeRS does not regulate SarA or *agr* (*16*−*18*).

Other *S. aureus* virulence regulators include the SarA-family members (SarR-SarV), SvrA, Rot, ArlRS, MgrA, and SrrAB (*19*−*28*).

Although these global regulators have been well studied in vitro, the behavior of these regulators in the in vivo

† We gratefully acknowledge USPHS Grant HL36611 from the National Heart, Lung, and Blood Institute and the Minnesota Medical Foundation for financial support. A.A.P. is supported by an NIAID Predoctoral Fellowship (Training Grant T32 AI 07421).

* To whom correspondence should be addressed.

‡ Department of Microbiology, University of Minnesota.

§ Department of Veterinary Biosciences, University of Minnesota.

[1] Abbreviations: *tst*/TSST-1, toxic shock syndrome toxin; *spa*, protein A; *ica*, intracellular adhesion locus; *agr*, accessory gene regulator; Sar, staphylococcal accessory regulator; Sae, staphylococcal accessory element; *gyr*, gyrase; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

10.1021/bi0603266 CCC: $37.00 
Published on Web 12/10/2006

infection environment has not been thoroughly studied. In particular, little is known about the mechanism used by these virulence regulators when stimulated by the environmental signals that *S. aureus* is known to respond to (*25*, *29*−*32*). For this reason, our laboratory began to study the staphylococcal respiratory response (SrrAB), a two-component system that regulates virulence factors. Creation of an *srrB* null mutant in strain RN4220 as well as subsequent gel shift analyses indicated that SrrAB regulates *agr*, *tst*, *spa*, *srr*, and *icaR* transcription. Furthermore, overexpression of SrrAB decreases virulence in two rabbit models of infection and decreases biofilm formation in a microtiter plate assay (*25*, *33*, *34*). Other investigators have found that an SrrAB knockout strain displayed changes in the expression of proteins involved in energy metabolism and other metabolic processes in response to oxygen availability (*35*). Our investigations with the *srrB* null mutant in strain RN4220 demonstrated that SrrAB acts in the global regulation of staphylococcal virulence factors, regulates virulence in vivo, and affects biofilm formation. Due to the limitations of studying genetic regulation with strain RN4220, we undertook the present study in order to characterize an *srrAB* null mutant in a clinical isolate, MN8. We used an inducible antisense vector to create an *srrA* antisense construct capable of significantly decreasing *srrA* and *srrB* RNA. We used RT-PCR analysis in order to verify the construction of our mutant as well as assess the transcription of RNAIII, *tst*, *spa*, *icaR*, and *icaA* in both mutant and wild-type strains grown aerobically and in low-oxygen conditions. In the work presented here, we found that induction of antisense transcription resulted in decreased levels of *tst*, *spa*, and *icaR* transcript in aerobic conditions. Our current work, combined with our previous studies using strain RN4220, indicates that SrrAB may differentially regulate virulence factors in aerobic and anaerobic conditions.

## EXPERIMENTAL PROCEDURES

*Strains and Growth Conditions. S. aureus* strain MN8 is a TSST-1 positive clinical isolate obtained from a case of menstrual TSS (*36*). The plasmid pYJY4 is a 6.6 kb antisense expression shuttle vector that utilizes the pE194 origin and $Erm^R$ for *S. aureus* growth, the pUC19 origin and $Ap^R$ for *E. coli* growth, and the inducible xylose/tetracycline chimeric promoter system for inducible antisense transcription (*37*). The region of *srrA* targeted with antisense RNA was chosen on the basis of its likelihood of being single stranded in the 20 most likely predicted foldings of *srrA*. A 468 bp PCR product corresponding to nucleotides 88−556 of srrA was cloned into pYJY4 in the antisense orientation using restriction enzymes *Nco*I and *Asc*I in order to make the inducible *srrA* antisense construct pYJY4::AS*srrA*. Plasmid pYJY4::AS*srrA* was transformed into *S. aureus* strain MN8 in order to create strain MN5000. A diagram of the *srrAB* locus and pYJY4::AS*srrA* is provided in Figure 1, and a list of all primers used in this study is provided in Table 1. S. *aureus* was propagated in Todd-Hewitt (TH) broth (Difco Laboratories, Sparks, MD) at 37 °C with appropriate selective or inducing antibiotics as indicated.

*Growth Curves. S. aureus* strains MN8(pYJY4) and MN5000 were grown overnight to stationary phase in TH broth with erythromycin (5 mg/mL) and molecular biology-grade tetracycline (0.15 mg/mL) (Sigma-Aldrich Corp., St. Louis, MO). Strain MN8 was grown overnight with tetracycline. Each strain was diluted 1:50 into 50 mL of fresh TH media in a 125 mL Erlenmeyer flask. Erythromycin was added to MN8(pYJY4) and MN5000 flasks. Each of the three cultures was split into two 25 mL cultures, with tetracycline added to one of the paired cultures for each strain. All strains were grown at 37 °C with shaking for 24 h, with samples taken every hour to monitor cell growth. Growth of the cultures was assessed by measuring the $OD_{600}$ at the designated times. The inoculating cultures were grown overnight in TH broth with erythromycin and tetracycline.

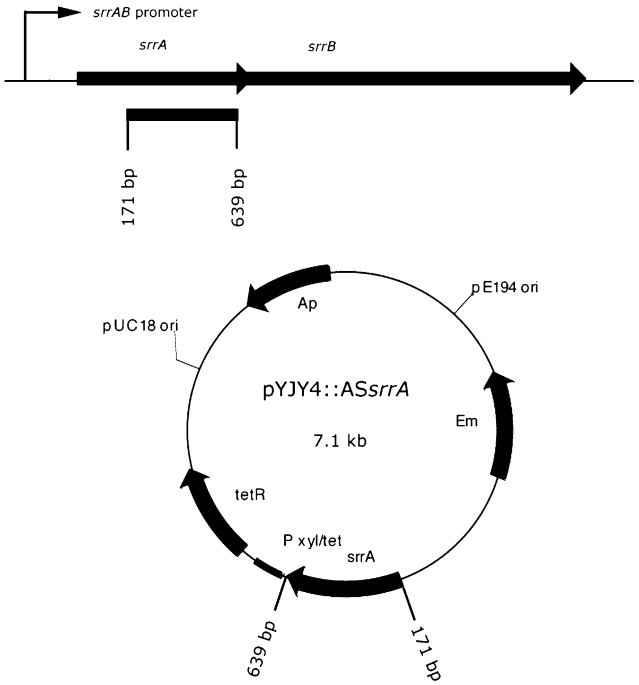


FIGURE 1: Diagram of the *srrAB* locus and the pYJY4::AS*srrA* construct. The *srrAB* locus, including the promoter region, is shown above. The 468 bp region (bp 171−639) of the 726 bp *srrA* that was targeted for antisense repression is indicated by a black bar below the locus. The gray bar in the N-terminal region of *srrA* indicates the portion of *srrA* used in RT-PCR analysis to quantify the *srrA* transcript. The *srrA*-specific probe used in northern analysis was PCR amplified as the full 726 bp *srrA* locus. The 7.1 kb pYJY4::AS*srrA* construct is diagramed below. The indicated 468 bp region of *srrA* was cloned downstream of the $P_{xyl/tet}$ promoter in the antisense orientation. Upon addition of tetracycline, the *tetR* repressor is turned off, allowing transcription from the $P_{xyl/tet}$ promoter.

*RNA Isolation.* Total RNA was acquired from the indicated strains of *S. aureus* using FastProtein Blue and the FastPrep 120 mechanical shearing instrument (Q-BIOgene, Irvine, CA), followed by isolation and purification using the RNeasy mini kit (Qiagen, Valencia, CA). DNA was removed from the samples using the DNA*free* kit (Ambion, Inc., Austin, TX) followed by two successive LiCl precipitations. RNA was quantified by spectrophotometric analysis ($\lambda = 260$ nm).

*Northern Analysis.* Northern blots were accomplished using a double-stranded DNA probe specific for full-length *srrA* transcript, as described in Figure 1 and Table 1. The *srrA* probe was made using the PCR DIG probe synthesis kit following the manufacturer's instructions (Roche Applied Science, Indianapolis, IN). Electrophoresis, blotting, hybridization, and detection of the DIG-labeled probe were accomplished according to the manufacturer's instructions in the DIG Application Manual (Roche Applied Science),

Table 1: Primers Used in This Study

| name | description | primer sequences | $T_{anneal}$[a] (°C) |
|---|---|---|---|
| *srrA* antisense construct | PCR amplification of antisense insert for pYJY4 | CGCCCATGGCACACGATTTAACTTTTCTCTAAGTCGTTTAACA ATAGGCGCGCCTGAAATGGATGGTATCCAGGT | |
| northern probe | *srrA* northern probe | CGCGGATCCATGTATTTATCACAAAGTTTGA ATGCGCAGCTTCACTAGACACA | |
| gyrA | *gyrA* qPCR | TGCGATTTCGTTCAGAGAAG ACGGCCTAAAGGACGTAATG | 60.5 |
| srrA | srrA *qPCR* | AGCATGTGTGGGAGGTATGA CCTCTTGGCCATTACTTGCTT | 60.4 |
| *srrB* | *srrB* qPCR | AGCCGGCTAAATAGTGTCGT ATGGCATTTTCGGTTTCTTG | 60.9 |
| *tst* | *tst* qPCR | TCGTAAGCCCTTTGTTGCTTG TCGTTTGTAGATGCTTTTGCAGTT | 62.5 |
| spa | spa *qPCR* | GACGGCAACAAGCCTGGTAA GCCGTTTGCTTTTGCAATGT | 62.6 |
| RNAIII | RNAIII qPCR | TTTTCAATCTATTTTTGGGGATGTT CCTTGGACTCAGTGCTATGTATTTTT | 59 |
| *icaR* | *icaR* qPCR | TCGAACTATTCAATTGATGCTTTA CAGAAAATTCCTCAGGCGTA | 57.5 |
| *icaA* | *icaA* qPCR | GCCATGTGTTGGATGTTGGT AACCTTTTCGTTTTCATTGTGCT | 59 |

[a] Annealing temperature is provided for primer sets used in qRT-PCR analysis.

using standard techniques (*38*). A 1.5% agarose−formaldehyde gel was used to electrophores the DIG-labeled RNA molecular weight markers as well as 5 $\mu$g of RNA from each sample in MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 2 mM EDTA, pH 7.0) for 6 h. Capillary transfer onto a positively charged nylon membrane was performed using 20 × SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0) overnight. Subsequent prehybridization and hybridization of the DIG-labeled probe was accomplished using DIG Easy Hyb. Hybridized probe was detected using an anti-DIG alkaline phosphatase conjugate antibody and chemiluminescent detection, according to the manufacturer's instructions. Due to the double-stranded nature of the full-length *srrA* probe, both antisense and sense *srrA* transcripts were identified by northern analysis. rRNA was used as a loading control and enabled quantification of RNA expression using ImageJ software (*39*).

*Western Analysis.* Western analysis was accomplished according to standard procedures (*38*). Briefly, *S. aureus* cultures were grown as indicated in the text and pelleted, and the supernatants were removed. The cell pellet was resuspended in phosphate-buffered saline (PBS, 5 mM $NaPO_4$, 0.15 M NaCl, pH 7.2), sonicated to lyse the cells, and centrifuged to separate the cell wall/membrane fraction from the cytoplasmic fraction. The protein in the cytoplasmic fractions was quantitated (Bio-Rad, Hercules, CA), and an identical amount of protein was loaded in each lane of the western analysis. Samples were electrophoresed on a 12% sodium dodecyl sulfate−polyacrylamide gel (SDS−PAGE) and blotted overnight onto a PVDF membrane. The primary SrrA antibody was a rabbit polyclonal antibody made in our laboratory (*33*). Detection was accomplished with an anti-rabbit secondary antibody conjugated to alkaline phosphatase (Sigma-Aldrich Corp., St. Louis, MO) and chromogenic detection. A *S. aureus* polyclonal antibody (AbD Serotec, Kingston, NH) was used as a loading control.

*Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR).* Two-step qRT-PCR was employed in order to minimize sample-to-sample variability in the efficiency of reverse transcription. Approximately 2 $\mu$g of RNA was reverse transcribed with Superscript II using random hexamer primers (Invitrogen, Carlsbad, CA). This reaction provided the DNA that was used in subsequent qPCR reactions. Each qPCR reaction using the QuantiTect SYBR Green PCR kit employed 3 $\mu$L of DNA and 21 pmol of each primer, according to the manufacturer's instructions (Qiagen, Valencia, CA). The reactions were performed using an iCycler machine (Bio-Rad, Hercules, CA). Reactions were performed at 95 °C for 15 min followed by 40 cycles of 95 °C for 20 s, annealing temperature for 20 s, and 72 °C for 30 s. The primers used to quantify *gyrA*, *srrA*, *srrB*, *tst*, *spa*, RNAIII, *icaR*, and *icaA* as well as the annealing temperatures used in each reaction are detailed in Table 1. The data were analyzed using the $\Delta\Delta Ct$ method (*40*). Briefly, the *Ct* value for each gene was subtracted from the *gyrA Ct* value for that same sample in order to obtain the $\Delta Ct$ value. The $\Delta Ct$ values of one sample were subtracted from the $\Delta Ct$ values of another sample in order to calculate the $\Delta\Delta Ct$ values. The $\Delta\Delta Ct$ value for each gene represents the change in expression of that particular gene between the two samples used in the comparison. Fold differences in RNA expression were obtained by calculating $2^{-\Delta\Delta Ct}$ for each gene. Fold differences of <1 were converted into meaningful values by calculating the negative inverse. During each experiment, the data were validated by calculating the PCR efficiency and correlation for a serial dilution of sample. PCR efficiencies of 80%−105% and correlations of 0.98 or greater were used. Unless otherwise noted, the data reported represent five independent experiments.

*Wiffle Ball Fluid.* Sterile wiffle balls approximately the size of golf balls were surgically implanted in the flanks of 10 Dutch-belted rabbits. The rabbits were allowed to heal over 6 weeks, during which time the wiffle balls filled with a sterile fluid transudate. Approximately 1 mL of wiffle ball fluid was removed aseptically from each rabbit and frozen for later use. The research presented here has complied with all relevant federal guidelines and institutional policies regarding animal use.

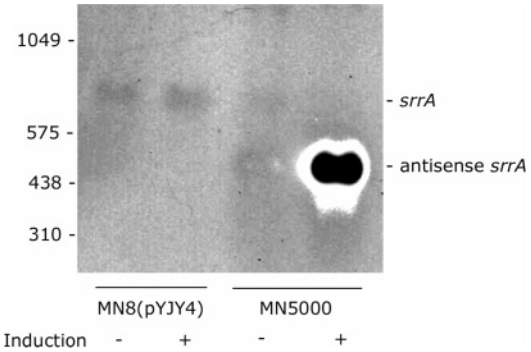


FIGURE 2: Northern analysis for *srrA*. Total RNA from uninduced and induced MN8(pYJY4) and MN5000 was subjected to northern analysis using a double-stranded DNA probe for full-length *srrA*. *srrA* appears as an approximately 700 bp band in both MN8(pYJY4) lanes as well as the uninduced MN5000 lane. Antisense *srrA* appears as an approximately 500 bp band in the induced MN5000 lane. The appearance of antisense *srrA* corresponds with the disappearance of *srrA* in the induced MN5000 lane.

*RT-PCR Amplification Products.* Representative PCR products for each of the transcripts tested were electrophoresed on an agarose gel in order to confirm the presence of a single amplification product of the desired length. Each of the products demonstrated a single band of the appropriate size (data not shown).

## RESULTS

*Creation of the Antisense srrA Strain MN5000.* As strain RN4220 is a randomly mutated laboratory strain and therefore not ideal for in vivo work, we sought to create an *srrAB* knockout strain in a clinical isolate. We chose to use strain MN8 because it is a well-studied clinical isolate from a case of menstrual TSS. Additionally, it expresses toxic shock syndrome toxin 1 (TSST-1), an important member of the *srrAB* regulon, as established in RN4220. Due to the difficulty of accomplishing genetic manipulations in clinical isolates, we chose to use the inducible antisense vector pYJY4. This plasmid has been used successfully to study virulence in *S. aureus* (*37*, *41*, *42*). We chose to target the *srrA* transcript as it is the first gene in the *srr* operon, and the *srrA* transcript is estimated to be 10 times more abundant than the *srrB* transcript. As *srrB* is not transcribed independently of *srrA*, we felt that the antisense RNA-mediated downregulation of the *srrA* transcript would also downregulate the *srrB* transcript, as the two are physically joined. Strain MN5000 was created by transformation of *S. aureus* MN8 with pYJY4::AS*srrA*, which was created as described above and in Figure 1 and Table 1.

*Downregulation of srrA Transcript in MN5000.* Northern blot analysis using a double-stranded DNA probe to full-length *srrA* was used to optimize the concentration of tetracycline for antisense induction, verify antisense RNA transcription, and demonstrate sense *srrA* transcript repression. Strain MN5000 and a vector control, grown with and without induction, were subjected to northern analysis using an *srrA* probe. The vector control strain MN8(pYJY4) as well as uninduced MN5000 demonstrated the presence of the *srrA* transcript at approximately 700 bp. Induced MN5000 demonstrated strong induction of antisense *srrA* RNA at approximately 500 bp and a corresponding absence of *srrA* at 700 bp (Figure 2). Uninduced MN5000 demonstrated the same level of *srrA* expression as uninduced MN8, when the results were normalized to the rRNA control. Tetracycline at a concentration of 0.15 $\mu$g/mL was the lowest concentration of antibiotic that provided maximal transcription of antisense *srrA* (data not shown).

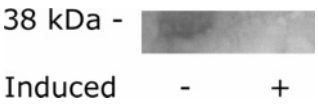


FIGURE 3: Western analysis for SrrA. Cytoplasmic extracts of MN5000 grown with and without induction were prepared as described in the text. Western analysis for SrrA revealed that induction of antisense *srrA* in MN5000 results in a decrease in SrrA protein expression.

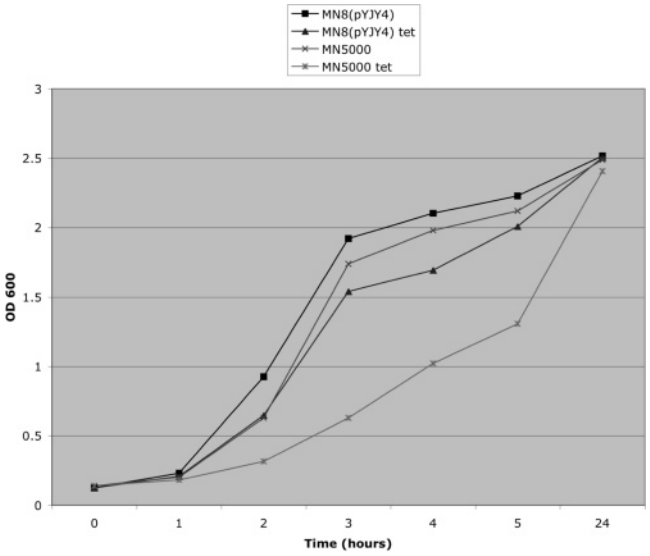


FIGURE 4: Growth curves of uninduced and induced MN8(pYJY4) and MN5000. The induction of antisense *srrA* in MN5000 resulted in a growth defect, compared to uninduced MN5000 and MN8-(pYJY4) with and without induction. There was an approximately 2 h lag between the entry of uninduced MN5000 into the postexponential phase and the entry of induced MN5000 into the postexponential phase.

*Downregulation of SrrA in MN5000.* In order to demonstrate that downregulation of *srrA* RNA results in downregulation of SrrA expression, western analysis of induced and uninduced MN5000 was performed. Strains were grown to postexponential phase, and the cytoplasmic expression of SrrA was assessed. Uninduced MN5000 demonstrated the presence of SrrA, while antisense induction of MN5000 resulted in an undetectable amount of SrrA (Figure 3). These data indicate that repression of *srrA* transcript in MN5000 results in repression of SrrA expression as well.

*Induced MN5000 Has a Growth Defect.* In order to assess the growth of our *srrA* downregulated strain, growth curves were performed using strains MN8(pYJY4) and MN5000 grown with and without tetracycline. In each case, the strains were grown overnight to stationary phase in the presence of tetracycline. Induced MN5000 exhibited a significant growth defect compared to uninduced MN5000, reaching postexponential phase approximately 2 h after the uninduced culture (Figure 4). The addition of tetracycline to the vector control culture did slightly inhibit growth. The final cell density in stationary phase of induced MN5000 recovered to near-uninduced levels, likely as the result of the overnight breakdown or consumption of tetracycline in the culture. The

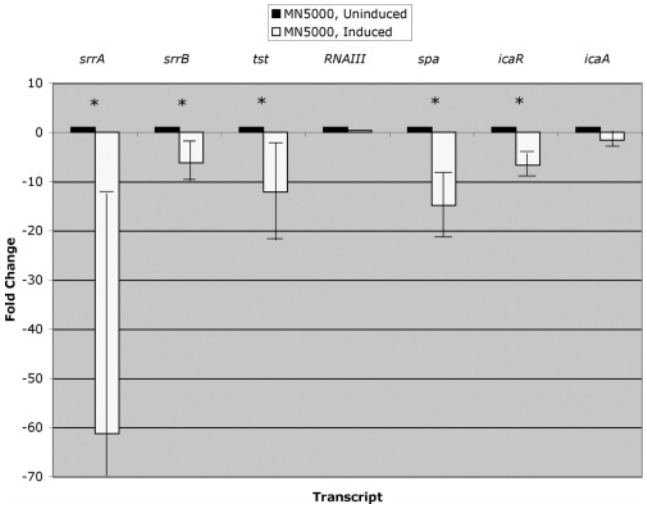


FIGURE 5: Aerobic growth in TH broth: uninduced vs induced MN5000. Samples were subjected to RT-PCR analysis as described in the text. Differences in expression were noted when the standard deviation of the mean was more than 2-fold changed, compared to the average of the control. The asterisk indicates significant differences in expression. Induction of antisense expression in MN5000 grown aerobically in TH broth resulted in a significant decrease in *srrA*, *srrB*, *tst*, *spa*, and *icaR* transcript levels.

antisense vector itself did not appear to inhibit growth significantly when induced and uninduced MN8 were compared to MN8(pYJY4) (data not shown). These results show that the downregulation of *srrA* in induced MN5000 causes a growth defect. Although we cannot rule out the possibility that transcription of antisense *srrA* is a metabolic burden that slows bacterial growth, this possibility seems unlikely. Other investigators have reported a growth defect in SrrAB knockout strains, particularly in anaerobic conditions (*25*, *34*, *35*).

*Comparison of Virulence Gene Expression in MN5000.* Upon reaching postexponential phase, samples were removed from uninduced and induced MN5000 grown aerobically in TH broth. RNA was obtained and reverse transcribed as described above. Quantitative PCR was used to demonstrate the repression of *srrAB* transcript upon induction of MN5000, as well as assess the impact of *srrAB* repression on the levels of *tst*, RNAIII, *spa*, *icaR*, and *icaA* transcript (Figure 5). Results were considered significant when the mean and standard deviations of the five independent experiments were both more than 2-fold increased or decreased, compared to the average of the control. The values of the five independent experiments were derived by averaging the three replicates performed for each independent experiment. Upon induction of antisense transcription in an aerobic TH broth culture, *srrA* was approximately 61-fold repressed and *srrB* was 6-fold repressed. Upon repression of *srrAB*, *tst* (12-fold), *spa* (15-fold), and *icaR* (7-fold) were also repressed. RNAIII and *icaA* were not significantly up- or downregulated. These results are consistent with our previous work, which indicated that SrrAB played a role in *tst*, *spa*, *agr*, and *icaR* transcription but not *icaA* transcription (*25*, *33*, *34*). It is surprising that RNAIII expression was not affected. These results indicate that SrrAB is required for *tst*, *spa*, and *icaR* expression at wild-type levels in rich media under aerobic conditions.

We next sought to compare the expression of uninduced MN5000 in TH broth grown aerobically vs TH broth grown

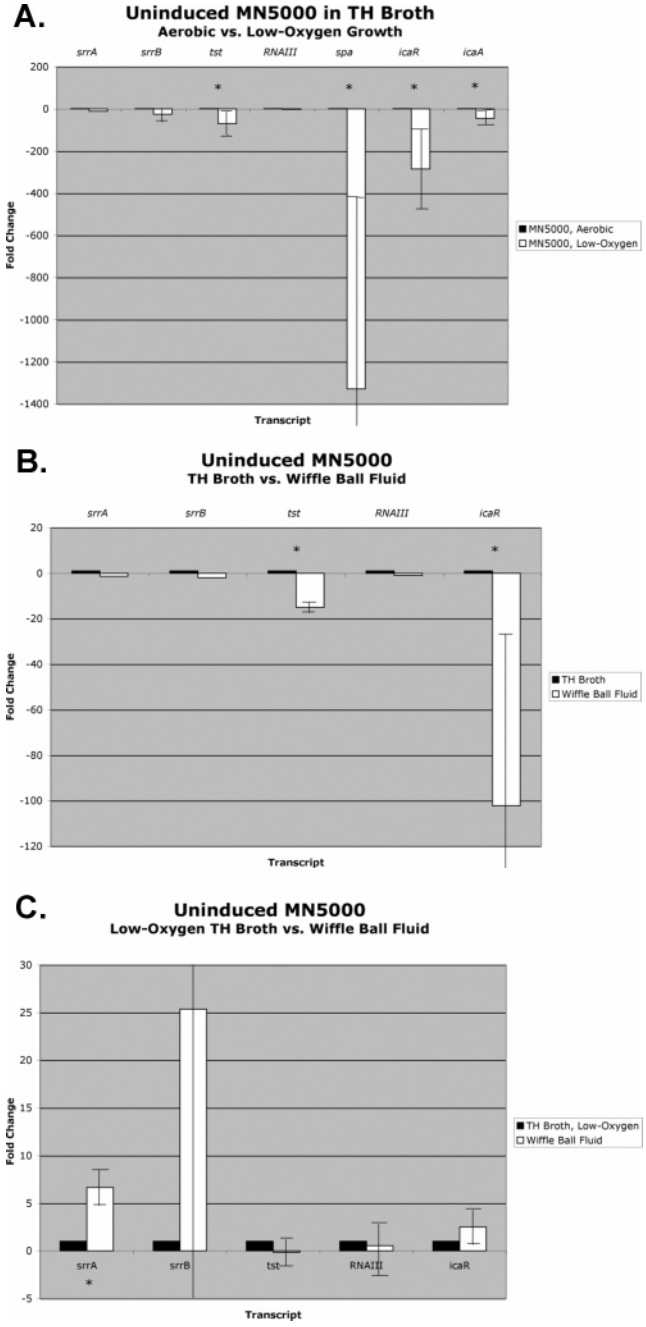


FIGURE 6: Uninduced MN5000. Samples were subjected to RT-PCR analysis as described in the text. Differences in expression were noted when the standard deviation of the mean was more than 2-fold changed, compared to the average of the control. The asterisk indicates significant differences in expression. (A) Comparison of uninduced MN5000 grown aerobically and with low oxygen in TH broth resulted in a significant decrease in *tst*, *spa*, *icaR*, and *icaA* transcript levels in low-oxygen conditions. (B) Comparison of uninduced MN5000 grown aerobically in TH broth and in wiffle ball fluid resulted in a significant decrease in *tst* and *icaR* transcript levels. (C) MN5000 grown in wiffle ball fluid resulted in a significant increase in *srrA* transcript levels compared to low-oxygen TH broth.

under low-oxygen conditions (~1% $O_2$). Our data showed that there was no change in *srrAB* transcription in low-oxygen conditions compared to typical aerobic growth (Figure 6). In spite of this, *tst* (69-fold), *spa* (1329-fold), *icaR* (284-fold), and *icaA* (45-fold) were repressed. These findings are consistent with the known oxygen-dependent regulation of *tst*/TSST-1 and *icaR* (*34*, *44*). To our knowl-

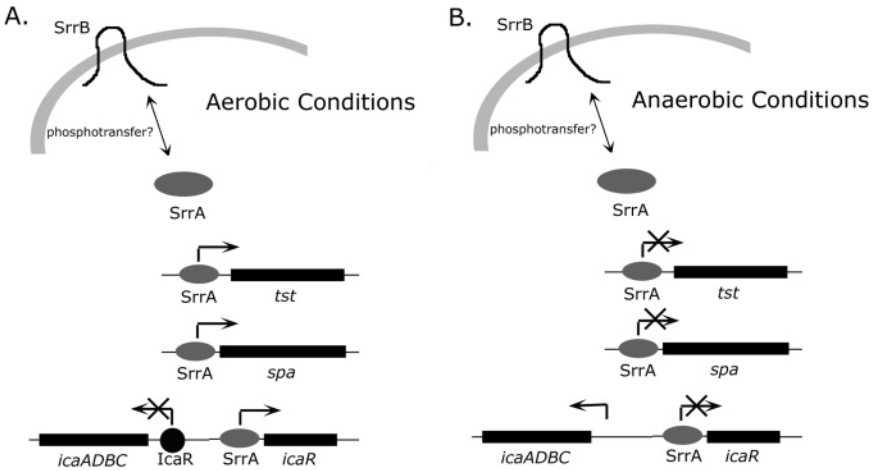


FIGURE 7: SrrAB model. (A) Under aerobic conditions, SrrAB is predicted to bind upstream of the *tst*, *spa*, and *icaR* promoters, resulting in transcriptional upregulation. Upregulation at these loci is predicted to increase TSST-1 and protein A levels and decrease biofilm formation, as described in the text. (B) Under anaerobic conditions or in nutrient-poor media, SrrAB is predicted to bind upstream of *tst*, *spa*, and *icaR* loci, resulting in a decrease in transcription at these loci. This is predicted to decrease TSST-1 and protein A levels while enhancing biofilm formation, as described in the text. At the present time the identity of the signal that SrrB recognizes is unknown, as is the condition (aerobic or anaerobic) in which SrrA is preferentially phosphorylated.

edge, this is the first report that *spa* is regulated in an oxygen-dependent manner. These data indicate that oxygen alone does not alter the expression of *srrAB* but that *tst*, *spa*, *icaR*, and *icaA* transcripts are repressed in a low-oxygen environment when the *srrAB* locus is intact. Signaling through the SrrAB system may regulate expression at these loci in response to oxygen availability.

We then assessed expression in TH broth under low-oxygen conditions using uninduced MN5000. Our data (Figure 6A) indicate that oxygen itself does not alter transcription of *srrAB*, indicating that other factors in the low-oxygen environment must be responsible for *srrAB* regulation.

We next sought to compare the transcription profiles of uninduced MN5000 grown aerobically in TH broth with uninduced MN5000 grown in wiffle ball fluid (WBF). Due to the small amount of wiffle ball fluid that can be obtained from each rabbit, data presented on bacteria grown in WBF represent the average of two independent experiments. Comparison of aerobic TH broth and WBF showed that WBF does not alter the transcription of *srrAB* (Figure 6B). WBF did repress *tst* and *icaR* transcripts, compared to aerobically grown MN5000 in TH broth. Although these data do not suggest a mechanism for the repression of *tst* and *icaR* transcripts, both are known to be regulated by SrrAB, which is intact in this experiment. We then compared the transcriptional profiles of uninduced MN5000 grown in TH broth with a low-oxygen environment to uninduced MN5000 grown in WBF. In this instance, *srrA* transcript was significantly increased in WBF compared to low-oxygen TH broth (Figure 6C). The *srrB* transcript was not significantly altered due to the relatively decreased expression of *srrB* compared with *srrA* and the standard deviation of these replicates. Other transcripts (*srrB*, *tst*, RNAIII, *icaR*) were not significantly altered. These data indicate that WBF is most closely mimicked by TH broth in a low-oxygen environment. In addition, an environmental factor found in the WBF but not low-oxygen TH broth may be responsible for increasing *srrA* transcript.

## DISCUSSION

Previous work on the SrrAB system has indicated that SrrAB regulates *agr* RNAIII, TSST-1, protein A, and IcaR in response to oxygen availability by acting at the level of transcription. In this study we sought to confirm that SrrAB regulates transcript levels using RT-PCR to study the transcriptional profiles of *S. aureus* MN5000 grown in TH broth in both aerobic and low-oxygen conditions. In order to assess this regulation in a clinical isolate, we employed an inducible antisense vector, pYJY4, which has been used successfully in the past to study virulence in animal models (*44*, *45*).

Northern and western analyses of uninduced and induced MN5000 showed that *srrA* antisense RNA was able to repress *srrA* transcript levels as well as SrrA protein levels. When grown aerobically in rich media, induction of the antisense RNA resulted in a growth defect. The time necessary to reach postexponential phase was 2 h longer when antisense induction occurred. Growth in TH media with a low-oxygen environment (1% $O_2$) resulted in a similar growth defect. While it is possible that production of antisense RNA can cause a growth defect, this possibility is unlikely. Growth defects due solely to production of antisense RNA have not been reported in the published literature. Furthermore, other authors have reported that SrrAB knock-outs demonstrate a growth defect, particularly in anaerobic conditions. Our experiments indicate that SrrAB-repressed strains possess a growth defect in aerobic as well as low-oxygen environments.

RT-PCR analysis of gene expression in MN5000 yielded complex results. In TH broth with an aerobic environment, *srrAB* repression due to antisense induction occurred. In these conditions, it was shown that SrrAB was necessary for transcription of *tst*, *spa*, and *icaR* at wild-type levels. Growth in TH broth in a low-oxygen environment demonstrated that oxygen alone did not increase or decrease *srrAB* transcript levels in rich media. In TH broth with a low-oxygen environment, *tst*, *spa*, *icaR*, and *icaA* transcripts were repressed when *srrAB* expression was intact. The repression of *tst* and *icaR* transcripts is known to occur in low-oxygen

conditions. This is the first report of the *spa* transcript being regulated in response to environmental oxygen.

When MN5000 was not induced, *srrA* expression was found to be slightly higher in WBF compared to low-oxygen TH broth. Comparison of WBF with aerobic TH broth demonstrated no difference in *srrA* expression. The best model of gene expression in WBF appears to be TH broth in a low-oxygen environment, although a factor found in WBF appears to be able to increase *srrA* transcript levels above those found in low-oxygen TH broth.

These data have led to a more detailed and nuanced understanding of the role of SrrAB in virulence factor regulation. Our current model of SrrAB signal transduction incorporates aerobic and anaerobic growth, as well as the known role of SrrAB in regulating proteins involved in energy metabolism (Figure 7). In aerobic conditions, SrrAB activates transcription of *tst*, *spa*, and *icaR* by binding to promoter regions. This increases TSST-1 and protein A production, enhancing virulence in an oxygen-rich environment. Increasing IcaR, which is a transcriptional repressor of *icaADBC*, decreases transcription of the structural genes of the *ica* locus. This decreases *ica*-related biofilm formation, which is known to occur preferentially in low-oxygen conditions. Under anaerobic conditions, transcription of *srrAB* is enhanced. The role of SrrAB in enhancing expression of alcohol dehydrogenase and lactate dehydrogenase, which are important in anaerobic growth, has been described previously (*35*). Under anaerobic conditions, SrrAB represses transcription of *tst*, *spa*, and *icaR*. This not only decreases production of "accessory" virulence factors when energy is limiting but also relieves the IcaR-mediated repression of *icaADBC* transcription. This allows for the enhanced *ica*-mediated biofilm formation in *S. aureus* under anaerobic conditions. Repression vs activation of *tst*, *spa*, and *icaR* transcription may be regulated by the phosphorylation state of SrrA, the relative affinity of SrrA or phosphorylated SrrA for promoter DNA, and the location of the binding sites within the promoter.

In this paper, we have demonstrated the utility of using an inducible antisense vector to repress a virulence regulator and study virulence gene expression in a *S. aureus* clinical isolate. This is the first report of an inducible antisense construct being used to repress a regulatory transcript. Inducing an antisense *srrA* transcript repressed *srrA* and the cotranscribed *srrB* RNA, as well as repressed SrrA protein expression in the cytoplasm. Using RT-PCR we were able to show that the intact *srrAB* system is needed to express *tst*, *spa*, and *icaR* transcripts at wild-type levels in rich media under aerobic conditions. Furthermore, expression of *srrAB* is necessary for low-oxygen growth or growth in nutrient-poor media, although transcription of *srrAB* is not induced by oxygen alone. This work has contributed to our understanding of virulence regulation by SrrAB by highlighting the differences in virulence factor regulation between aerobic and anaerobic conditions. Our data also indicate that WBF, a model fluid for the infection environment found in subcutaneous tissue, is most closely mimicked by TH broth in low-oxygen (1% $O_2$) conditions.

## ACKNOWLEDGMENT

The authors thank Sara M. Vetter and Rachel Ogilvie for helpful discussions of RT-PCR techniques and results. Sara M. Vetter and Tim Leonard are gratefully acknowledged for help with western blots and images, respectively.

## REFERENCES

1. Novick, R. P. (2000) in *Gram-Positive Pathogens* (Fischetti, V. A., Ed.) pp 392−407, American Society for Microbiology, Washington, DC.
2. Pragman, A. A., and Schlievert, P. M. (2004) Virulence regulation in *Staphylococcus aureus*: the need for in vivo analysis of virulence factor regulation, *FEMS Immunol. Med. Microbiol. 42*, 147−154.
3. Peng, H. L., Novick, R. P., Kreiswirth, B., Kornblum, J., and Schlievert, P. (1988) Cloning, characterization, and sequencing of an accessory gene regulator (agr) in *Staphylococcus aureus*, *J. Bacteriol. 170*, 4365−4372.
4. Recsei, P., Kreiswirth, B., O'Reilly, M., Schlievert, P., Gruss, A., and Novick, R. P. (1986) Regulation of exoprotein gene expression in *Staphylococcus aureus* by agr, *Mol. Gen. Genet. 202*, 58−61.
5. Goerke, C., Fluckiger, U., Steinhuber, A., Zimmerli, W., and Wolz, C. (2001) Impact of the regulatory loci agr, sarA and sae of *Staphylococcus aureus* on the induction of alpha-toxin during device-related infection resolved by direct quantitative transcript analysis, *Mol. Microbiol. 40*, 1439−1447.
6. Yarwood, J. M., McCormick, J. K., Paustian, M. L., Kapur, V., and Schlievert, P. M. (2002) Repression of the *Staphylococcus aureus* accessory gene regulator in serum and in vivo, *J. Bacteriol. 184*, 1095−1101.
7. Goerke, C., Campana, S., Bayer, M. G., Doring, G., Botzenhart, K., and Wolz, C. (2000) Direct quantitative transcript analysis of the agr regulon of *Staphylococcus aureus* during human infection in comparison to the expression profile in vitro, *Infect. Immun. 68*, 1304−1311.
8. Wesson, C. A., Liou, L. E., Todd, K. M., Bohach, G. A., Trumble, W. R., and Bayles, K. W. (1998) *Staphylococcus aureus* Agr and Sar global regulators influence internalization and induction of apoptosis, *Infect. Immun. 66*, 5238−5243.
9. Qazi, S. N., Counil, E., Morrissey, J., Rees, C. E., Cockayne, A., Winzer, K., Chan, W. C., Williams, P., and Hill, P. J. (2001) agr expression precedes escape of internalized *Staphylococcus aureus* from the host endosome, *Infect. Immun. 69*, 7074−7082.
10. Chan, P. F., and Foster, S. J. (1998) Role of SarA in virulence determinant production and environmental signal transduction in *Staphylococcus aureus*, *J. Bacteriol. 180*, 6232−6241.
11. Heinrichs, J. H., Bayer, M. G., and Cheung, A. L. (1996) Characterization of the sar locus and its interaction with agr in *Staphylococcus aureus*, *J. Bacteriol. 178*, 418−423.
12. Chien, Y., and Cheung, A. L. (1998) Molecular interactions between two global regulators, sar and agr, in *Staphylococcus aureus*, *J. Biol. Chem. 273*, 2645−2652.
13. Rechtin, T. M., Gillaspy, A. F., Schumacher, M. A., Brennan, R. G., Smeltzer, M. S., and Hurlburt, B. K. (1999) Characterization of the SarA virulence gene regulator of *Staphylococcus aureus*, *Mol. Microbiol. 33*, 307−316.
14. Cheung, A. L., Bayer, M. G., and Heinrichs, J. H. (1997) sar genetic determinants necessary for transcription of RNAII and RNAIII in the agr locus of *Staphylococcus aureus*, *J. Bacteriol. 179*, 3963−3971.
15. Wolz, C., Pohlmann-Dietze, P., Steinhuber, A., Chien, Y. T., Manna, A., van Wamel, W., and Cheung, A. (2000) Agr-independent regulation of fibronectin-binding protein(s) by the regulatory locus sar in *Staphylococcus aureus*, *Mol. Microbiol. 36*, 230−243.
16. Giraudo, A. T., Raspanti, C. G., Calzolari, A., and Nagel, R. (1994) Characterization of a Tn*551*-mutant of *Staphylococcus aureus* defective in the production of several exoproteins, *Can. J. Microbiol. 40*, 677−681.
17. Giraudo, A. T., Calzolari, A., Cataldi, A. A., Bogni, C., and Nagel, R. (1999) The sae locus of *Staphylococcus aureus* encodes a two-component regulatory system, *FEMS Microbiol. Lett. 177*, 15−22.
18. Giraudo, A. T., Cheung, A. L., and Nagel, R. (1997) The sae locus of *Staphylococcus aureus* controls exoprotein synthesis at the transcriptional level, *Arch. Microbiol. 168*, 53−58.
19. Tegmark, K., Karlsson, A., and Arvidson, S. (2000) Identification and characterization of SarH1, a new global regulator of virulence gene expression in *Staphylococcus aureus*, *Mol. Microbiol. 37*, 398−409.

20. Manna, A., and Cheung, A. L. (2001) Characterization of sarR, a modulator of sar expression in *Staphylococcus aureus*, *Infect. Immun. 69*, 885−896.
21. Schmidt, K. A., Manna, A. C., Gill, S., and Cheung, A. L. (2001) SarT, a repressor of alpha-hemolysin in *Staphylococcus aureus*, *Infect. Immun. 69*, 4749−4758.
22. Manna, A. C., and Cheung, A. L. (2003) sarU, a sarA homolog, is repressed by SarT and regulates virulence genes in *Staphylococcus aureus*, *Infect. Immun. 71*, 343−353.
23. Manna, A. C., Ingavale, S. S., Maloney, M., van Wamel, W., and Cheung, A. L. (2004) Identification of sarV (SA2062), a new transcriptional regulator, is repressed by SarA and MgrA (SA0641) and involved in the regulation of autolysis in *Staphylococcus aureus*, *J. Bacteriol. 186*, 5267−5280.
24. Said-Salim, B., Dunman, P. M., McAleese, F. M., Macapagal, D., Murphy, E., McNamara, P. J., Arvidson, S., Foster, T. J., Projan, S. J., and Kreiswirth, B. N. (2003) Global regulation of *Staphylococcus aureus* genes by Rot, *J. Bacteriol. 185*, 610−619.
25. Yarwood, J. M., McCormick, J. K., and Schlievert, P. M. (2001) Identification of a novel two-component regulatory system that acts in global regulation of virulence factors of *Staphylococcus aureus*, *J. Bacteriol. 183*, 1113−1123.
26. Fournier, B., and Hooper, D. C. (2000) A new two-component regulatory system involved in adhesion, autolysis, and extracellular proteolytic activity of *Staphylococcus aureus*, *J. Bacteriol. 182*, 3955−3964.
27. Luong, T. T., Newell, S. W., and Lee, C. Y. (2003) Mgr, a novel global regulator in *Staphylococcus aureus*, *J. Bacteriol. 185*, 3703−3710.
28. Garvis, S., Mei, J. M., Ruiz-Albert, J., and Holden, D. W. (2002) *Staphylococcus aureus* svrA: a gene required for virulence and expression of the agr locus, *Microbiology 148*, 3235−3243.
29. Wong, A. C., and Bergdoll, M. S. (1990) Effect of environmental conditions on production of toxic shock syndrome toxin 1 by *Staphylococcus aureus*, *Infect. Immun. 58*, 1026−1029.
30. Kass, E. H., Kendrick, M. I., Tsai, Y. C., and Parsonnet, J. (1987) Interaction of magnesium ion, oxygen tension, and temperature in the production of toxic-shock-syndrome toxin-1 by *Staphylococcus aureus*, *J. Infect. Dis. 155*, 812−815.
31. Chan, P. F., and Foster, S. J. (1998) The role of environmental factors in the regulation of virulence-determinant expression in *Staphylococcus aureus*, *Microbiology 144*, 2469−2479.
32. Ross, R. A., and Onderdonk, A. B. (2000) Production of toxic shock syndrome toxin 1 by *Staphylococcus aureus* requires both oxygen and carbon dioxide, *Infect. Immun. 68*, 5205−5209.
33. Pragman, A. A., Yarwood, J. M., Tripp, T. J., and Schlievert, P. M. (2004) Characterization of virulence factor regulation by SrrAB, a two-component system in *Staphylococcus aureus*, *J. Bacteriol. 186*, 2430−2438.
34. Timpert, P., Cramton, S. E., Ulrich, M., Pragman, A. A., Kraenzler, H., Ziebuhr, W., Wolz, C., Schlievert, P. M., and Doring, G. (2005) Differential gene regulation in *Staphylococcus aureus* under aerobic and anaerobic growth, *Mol. Microbiol.* (in review).
35. Throup, J. P., Zappacosta, F., Lunsford, R. D., Annan, R. S., Carr, S. A., Lonsdale, J. T., Bryant, A. P., McDevitt, D., Rosenberg, M., and Burnham, M. K. (2001) The srhSR gene pair from *Staphylococcus aureus*: genomic and proteomic approaches to the identification and characterization of gene function, *Biochemistry 40*, 10392−10401.
36. Blomster-Hautamaa, D. A., and Schlievert, P. M. (1988) Preparation of toxic shock syndrome toxin-1, *Methods Enzymol. 165*, 37−43.
37. Yin, D., and Ji, Y. (2002) Genomic analysis using conditional phenotypes generated by antisense RNA, *Curr. Opin. Microbiol. 5*, 330−333.
38. Sambrook, J., and Russel, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
39. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-ΔΔC(T)) Method, *Methods 25*, 402−408.
40. Abramoff, M. D., Magelhaes, P. J., and Ram, S. J. (2004) Image processing with image, *J. Biophotonics Int. 11*, 36−42.
41. Ji, Y., Woodnutt, G., Rosenberg, M., and Burnham, M. K. (2002) Identification of essential genes in *Staphylococcus aureus* using inducible antisense RNA, *Methods Enzymol. 358*, 123−128.
42. Ji, Y., Yin, D., Fox, B., Holmes, D. J., Payne, D., and Rosenberg, M. (2004) Validation of antibacterial mechanism of action using regulated antisense RNA expression in *Staphylococcus aureus*, *FEMS Microbiol. Lett. 231*, 177−184.
43. Yarwood, J. M., and Schlievert, P. M. (2000) Oxygen and carbon dioxide regulation of toxic shock syndrome toxin 1 production by *Staphylococcus aureus* MN8, *J. Clin. Microbiol. 38*, 1797−1803.
44. Ji, Y., Marra, A., Rosenberg, M., and Woodnutt, G. (1999) Regulated antisense RNA eliminates alpha-toxin virulence in *Staphylococcus aureus* infection, *J. Bacteriol. 181*, 6585−6590.
45. Ji, Y., Zhang, B., Van, S. F., Horn, Warren, P., Woodnutt, G., Burnham, M. K., and Rosenberg, M. (2001) Identification of critical staphylococcal genes using conditional phenotypes generated by antisense RNA, *Science 293*, 2266−2269.

BI0603266