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Original Research

Regulation of Staphylococcal Superantigen-Like Gene, *ssl8*, Expression in *Staphylococcus aureus* strain, RN6390

Madhulatha Pantrangi, PhD; Vineet K. Singh, PhD; and Sanjay K. Shukla, PhD

Staphylococcal superantigen-like (SSL) proteins, which are encoded by a cluster of eleven *ssl* genes, contribute to the *Staphylococcus aureus* virulence. Recently we reported *ssl8* expression profiles in seven clinically important strains—MW2, USA300FPR3757, MSSA476, Newman, RN6390, Mu50, and N315—and showed the differential expression of *ssl8* in Newman, RN6390, and USA300FPR3757 strains, despite harboring identical allelic forms of *ssl8,* suggesting the roles for different regulatory elements for this gene in different *S. aureus* strains. In this communication, using RN6390, a common laboratory *S. aureus* strain and its isogenic knockout mutant strains of *agr, sae, sarA*, *sigB, rot*, and the *agr*/*sigB*$^-$ double mutant, we showed that SarA and Rot are inducer and repressor, respectively, for *ssl8* expression in RN6390. This is in contrast to the Newman strain, where *ssl8* is positively regulated by Sae but negatively by Agr, indicating the variable expression of *ssl8* in clinical strains is more likely due to strain-specific regulatory elements.

Keywords: *Staphylococcus aureus; ssl8;* SarA; Rot; MRSA; Gene regulation

Pathogenesis of *Staphylococcus aureus* is complex and involves the coordinate expression of multiple toxin genes that are tightly controlled by global regulators at the transcription level.[1-3] These toxins are synthesized preferentially during the transition between exponential and stationary growth phases.[4] Among its large number of secreted toxins, is a group of staphylococcal superantigen-like (SSL) proteins encoded by 11 *ssl* (formerly known as staphylococcal exotoxin-like or *set*) genes.[5,6] Genes for *ssl* are located on a highly variable region called RD13 that corresponds to the *ssl* containing genomic islands (vSaα, SaPIn2, or SaPIm2) in *S. aureus* strains.[5,7]SSL proteins have limited overall sequence homology to the staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin 1 (TSST-1) and, thus, represent a novel family of exotoxin-like proteins.[6]

ssl genes are present in all *S. aureus* strains studied so far, suggesting a non-redundant role in bacterial survival.[5,8,9] The overall order of *ssl* genes is conserved, and the allelic variants of the same gene in different strains share 85% to 100% sequence similarity,[7] indicating the evidence of selective pressure during the infection. SSL proteins in the same strain have 36% to 67% sequence homology.[6,7] Although the SSL proteins have sequence homology and similar predicted structures,[5,6,8] each protein studied so far has a related but distinct function in host-pathogen interactions.[6,9-12] SSL proteins have been shown to help *S. aureus* to escape from the protective adaptive immune response of the host and thus may contribute to bacterial pathogenicity.[6,9-12] SSL5 interferes with the interaction between PSGL-1 (P-selectin glycoprotein ligand-1) and P-selectin and prevents neutrophil extravasations toward the site of infection.[11] SSL7 inhibits IgA dependent cellular effector functions mediated by FcαRI, such as phagocytosis, degranulation, and respiratory burst.[12] SSL7 and SSL9 interact with human antigen presenting cells (monocytes and dendritic cells) at independent active sites.[10] SSL11 induces

Corresponding Author: Sanjay K. Shukla, PhD; Center for Human Genetics; Marshfield Clinic Research Foundation; 1000 North Oak Avenue (MLR); Marshfield, WI 54449; Tel: (715) 389-5363; Email: shukla.sanjay@mcrf.mfldclin.edu

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Table 1. *S. aureus* wild type and mutant strains used in this study.

S. aureus Strains	Genotype description	References
RN6390	Laboratory strain related to 8325-4, *rsbU*⁻.	4
RN6911	RN6390 *agr*::*tetM*	23
AS5	RN6390 *sae*::Tn917	30
ALC488	RN6390 *sarA*::ermC	31
ALC1001	RN6390 *sigB::ermC*	32
PM783	RN6390 *rot*::*tetM*	15
VKS105 (double mutant)	RN6390Δ*agr*::*tetM* /*sigB*::*kan*^{r}	This study

proinflammatory cytokines from peripheral blood mononuclear cells.[13] SSL8 binds to tenascin C (TNC), a glycoprotein and inhibits TNC and fibronectin interaction and cell motility in keratinocytes.[14] *ssl8* gene seem to be more frequently present in *S. aureus* isolates causing atopic dermatitis.[15]

The success of *S. aureus* as a pathogen lies in its ability to exploit multiple virulence factors regulated by complex network of global gene regulators that includes but not limited to Agr (accessory gene regulator), SarA (staphylococcal accessory regulator), Sae (*S. aureus* exoprotein expression), SigB (sigma factor B) and Rot (repressor of toxins).[2-4,9,16,17] Recently, we reported the differential expression of *ssl8* in seven clinically relevant *S. aureus* isolates and showed that *ssl8* expression in the Newman strain is regulated by Sae and Agr.[16] In this brief communication, we show that the *ssl8* expression in the common laboratory strain, RN6390 is regulated differently than the Newman strain suggesting *ssl8* regulation is strain dependent.

Materials and Methods

Bacterial Strains, Media and Culture Conditions

The laboratory *S. aureus* strain RN6390 and its isogenic mutants used in this study are listed in table 1. For each experiment, *S. aureus* was grown either in tryptic soy broth (TSB) or on tryptic soy agar (TSA) plates (Beckton Dickinson) as needed. For gene expression studies, an overnight shaking culture, grown at 37°C in TSB was used to inoculate 50 ml of fresh TSB (dilution, 1:200). Bacterial growth was subsequently monitored by incubating the flasks at 37°C with constant shaking and measuring the turbidity of the culture every 30 min at OD_{600} using a spectrophotometer (Beckman Coulter, Inc., CA) until the cultures reached the stationary phase. There were no growth differences between RN6390 and mutant strains (data not shown).

Construction of an agr/sigB Knockout Mutant of RN6390

A *agr*/*sigB* mutant in RN6390 background was generated using a phage transduction procedure as described previously.[9]

RNA Extraction

For gene expression studies, both the RN6390 and the mutant strains were grown up to early-stationary phase (5.5 hours) and harvested immediately for RNA isolations. RNA was extracted using a Qiagen RNeasy Mini kit (Qiagen, Valencia, CA) by following the manufacturer's recommendations with a few modifications. Briefly, a 1.5 mL of bacterial culture was dispensed into 3 mL of RNA Protect (Qiagen Inc, CA) in a 15-mL tube and mixed by vortexing for 5 seconds followed by 5 minutes incubation at room temperature. The cells were pelleted by centrifuging at 1252*g* for 5 minutes followed by a wash with DEPC treated water and re-suspended into 1.0 mL of TRIzol reagent. The suspension was then transferred to a 2-mL Lysing Matrix tube (MP Biomedicals, Irvine, CA) and homogenized by shaking three times in the mini bead beater for 20 seconds at a speed of 6 m/s. The cells were incubated on ice for 1 minute in between each run. To the homogenate, 200 µL of RNA-grade chloroform was added and vortexed for 15 seconds, followed by a centrifugation at high speed for 5 minutes and carefully aspirated the upper layer. To this supernatant, 500 µL of RNA-grade isopropyl alcohol was added and mixed by inverting the tube several times. This sample (~700 µL) was then transferred to an RNeasy column and centrifuged for 15 seconds at ≥8000*g* and followed the manufacturer's guidelines for RNeasy cleanup protocol (Qiagen Inc, CA). Finally, RNA was eluted with 50 µL of DEPC-treated water. RNA concentration was determined by using Bio-Rad SmartSpec Plus Spectrophotometer (Analytical Instruments, LLC, MN). An aliquot of each RNA sample was electrophoresed on a 1.0% agarose gel to assess its integrity and quality.

Quantitative Real-Time PCR Analysis of ssl8 Expression

To quantify the *ssl8* transcript levels in RN6390 and its mutant strains, the isolated RNA samples were subjected to DNase treatment with Turbo DNA-free kit (Ambion, Austin, TX) and confirmed to be DNA free by PCR before cDNA synthesis. The first strand cDNA was prepared with 2 µg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Roche, Foster City, CA) according to the manufacturer's instructions.

From the above reaction mix, 2 µL (~100 to 200 ng) of cDNA template was further used for quantitative real-time PCR in the Light Cycler (Roche Diagnostics Corp, Indianapolis, IN). The reaction was carried out using TaqMan Universal PCR Master Mix (2X) (Applied Biosystems Inc, CA) and the

Table 2. Primers and probes used in this study.

Gene	Primer	Primer sequence (5' → 3')	Probe sequence	Reference
ssl8	Forward	CCAACAAAATCAAAAGTTCCA	FAM-CACATGGTTTAGATGT CTTTGCGGTACC-NFQ	This study
	Reverse	CACCACTAACACTAAATATTCTTCCA		This study
Gmk	Forward	ACTAGGGATGCGTTTGAAGCTTTAA	FAM-AAAGATGACCAATTT ATAG AATATG-NFQ	This study
	Reverse	ACCATAATAGTTGCCTACAT		This study
RNAIII	Forward	TCCATTTTACTAAGTCACCGATTGT	FAM-ATCTTGTGCCATTGAAATCACTCCTTCCTT-NFQ	33
	Reverse	TGTGATGGAAAATAGTTGATGAGTTGT		33

TaqMan Gene Expression Assays [which included PCR forward and reverse primers (900 nM/μL) and 6-FAM dye-labeled MGB probe (250 nM/μL) specific for *ssl8*, RNAIII and *gmk* individually] (Applied Biosystems Inc, CA) by following the manufacturer's instructions. The PCR primers and probes are listed in table 2.

Relative quantification of *ssl8,* RNAIII (target genes), and *gmk* (guanylate kinase; endogenous control) was performed using $2^{-\Delta\Delta C_T}$ calculation according to the manufacturer's guidelines (Roche Diagnostics Corp., Indianapolis, IN). This method compensates for the factors such as variability in cDNA synthesis and template concentration, and calculates transcript ratios (*ssl8*/*gmk* and RNAIII/*gmk*) rather than absolute values. However, the reliability of $2^{-\Delta\Delta C_T}$ calculation depends on the PCR efficiency. By validation experiment, we confirmed that the PCR efficiency for *ssl8,* RNAIII and *gmk* was approximately two, as expected. To quantify the *ssl8* and RNAIII expressions in our experiments, we used *gmk* as a reference gene.[13,19]. All the results were obtained from two independent cultures.

Results

RN6390 ssl8 Regulation by SarA and Rot

We have quantified the *ssl8* and RNAIII transcript levels in RN6390 and its mutant strains: RN6911 (RN6390 *agr*::*tetM*), AS5 (RN6390 *sae*::Tn917), ALC488 (RN6390 Δ*sarA*::*ermC*), ALC1001 (RN6390 Δ*sigB::ermC*), PM783 (RN6390 Δ*rot*::*tetM*) and VKS105 (Δ*agr*::*tetM* /*sigB*::*kan*^{r} double mutant of RN6390).

The *ssl8*/*gmk* transcript ratios in RN6390 (5.68×10^{-03}) were comparable to the ratios in *sae*$^-$ strain, AS5 (5.64×10^{-03}) and *agr*$^-$ strain, RN6911 (5.48×10^{-03}). The *ssl8*/*gmk* transcript

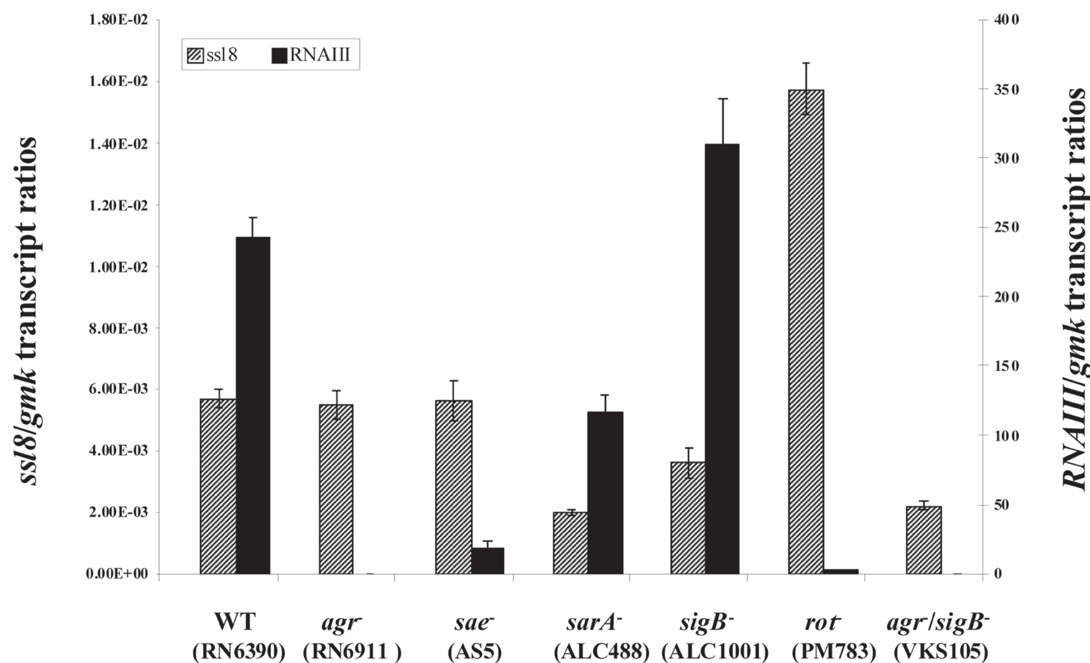

Figure 1. Transcript ratios of *ssl8/gmk* and RNAIII/*gmk*, quantified at early-stationary phase from RN6390 and its isogenic knockout mutants: RN6911 (RN6390 *agr*::*tetM*), AS5 (RN6390 *sae*::Tn917), ALC488 (RN6390 Δ*sarA*::ermC), ALC1001 (RN6390 Δ*sigB::ermC*), PM783 (RN6390 Δ*rot*::*tetM*) and VKS105 (Δ*agr*::*tetM* /*sigB*::*kan*^{r} double mutant of RN6390). The striped and dark bars indicate the *ssl8* (on primary axis) and RNAIII (on secondary axis) expression levels, respectively. Data represent the mean values of two independent measurements.

ratios in *sarA*- strain, ALC488 and *sigB*- strain, ALC1001 were 1.98×10^{-03} and 3.63×10^{-03}, respectively. In case of *rot*- strain, PM783 the ratio was 1.57×10^{-02} and in the double mutant, *agr*-/ *sigB*- strain VKS105, the ratio was 2.20×10^{-03} (figure 1).

The *sarA* and *rot* null mutations down-regulated (~3 fold) and up-regulated (~3 fold) *ssl8* expression, respectively, suggesting that the SarA is a positive regulator, whereas Rot is a negative regulator of *ssl8* expression in RN6390 (figure 1). However, the *sae* and *agr* mutations did not change the levels of *ssl8* transcripts, in contrast to *sigB* mutation, which caused ~1.5 fold decrease compared to the RN6390, suggesting SigB could be another positive regulator of *ssl8*. The double mutant, *agr/sigB* (VKS105) showed ~2.5 fold repression of *ssl8* compared to the RN6390, suggesting *agr* and *sigB* act in conjunction to regulate the *ssl8* expression.

Since Agr is one of the master regulators of exotoxins, we determined the transcript levels of RNAIII in these strains as well. The RNAIII/*gmk* transcript ratio in *sigB*- strain ALC1001 showed 1.2 fold increases compared to its wild type strain, RN6390. However, the RNAIII/*gmk* transcript ratios in *sarA*- (ALC488), *sae*- (AS5), and *rot*- (PM783) strains showed 2-, 13-, and 85-fold decreases, respectively, compared to the RN6390. As expected, the RNAIII levels were not detectable in *agr*- strains RN6911 (*agr*-) and VKS105 (*agr*-/*sigB*-). Since Agr does not seem to have a role in regulation of *ssl8* in RN6390, the increase and decrease in RNAIII levels did not show any corresponding up or down regulation of *ssl8* expression (figure 1).

Discussion

Staphylococcal exotoxins and SSL proteins significantly contribute to the *S. aureus* pathogenicity.[1,6,9-11,19-21] Their production is controlled by a network of global regulators, which act both positively and negatively depending on the virulence genes and host strains.[2-4,9,16,17] We showed here that *sarA* and *rot* mutations down-regulated and up-regulated *ssl8* expression, respectively, in RN6390, suggesting these two were the positive and negative regulators of *ssl8* in this strain (figure 1). SarA is a known positive regulator of a number of genes such as α- and δ-hemolysins, Agr response genes, *agrA-D*, *fnbA* and *fnbB* (fibronectin binding proteins), *splA*, *splB*, *splD* and *splF* (serine proteases), *ssl4,* and negative regulator of surface protein A (*spa*).[22] Similarly, a *sarA* homolog, *rot* is a known master regulator of a large number of genes as well. Using Affymetrix GeneChip based approach, Said-Salim et al showed that *rot* up regulates the expression of more than 80 genes (eg, coagulase, clumping factor B) and down regulates ~60 genes (eg, β-hemolysin, serine proteases).[9] In addition, we observed that *sigB* mutation also caused reduction in *ssl8* transcript levels but not to the extent of *sarA* mutant, indicating that SigB is a relatively weak positive regulator of *ssl8* in RN6390. In the *agr*-/*sigB*- double mutant, the expression of *ssl8* was down regulated. Similar down regulation of exotoxin genes has been shown in *sar*-/*agr*- double mutants compared to their parental and single mutant strains.[23] Our results indicated that Agr and SigB possibly act in concert to regulate *ssl8* expression. It was interesting to note that *ssl8* in the Newman strain was positively regulated by Sae and SigB and negatively by Agr, which is in contrast to RN6390.[18] Indeed, others have shown that *ssl7 and ssl11* are positively regulated by SaeRS system in Newman strain.[24] All of the Sae regulated genes were also found to be influenced by SigB but not always in the same direction.[25]

RNAIII levels do not seem to have any effect on *ssl8* expression in RN6390. However, it was interesting to note that RNAIII levels were highest in RN6390 compared to MW2, Newman, FPR3757, MSSA476, Mu50, and N315 due to deletion in *rsbU* gene of *sigB* operon,[18] suggesting the sigB has negative effect on RNAIII expression as has also been shown by others. Lauderdale et al[26] had reported that the *agr* RNAIII levels were elevated in the *sigB* mutants causing the anti-biofilm phenotypic effect. Unlike previously reported, we observed reduced RNAIII transcript levels in *sae*- strain of RN6390.[27] Curiously, RNAIII levels were also reduced in *rot*- strain of RN6390 (figure 1). Indeed, RNAIII interacts with *rot* mRNA and is involved in inhibition of *rot* mRNA translation.[28,29]

In summary, it appears that the expression and regulation of *ssl* in *S. aureus* is complicated and is determined by the strain specificity and not necessarily the common genetic background which is probably due to unrelated regulatory pathways. We showed here that the regulatory pathways of *ssl8* in RN6390 are different from our findings for the *ssl8* in the Newman strain[18] suggesting regulation of many individual virulence factors in *S. aureus* are strain-specific. This observation further highlights the complexity in regulation of virulence in *S. aureus* strains and poses challenges in considering all *S. aureus* as equal.

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Author Affiliations

Madhulatha Pantrangi, PhD,#; Vineet K Singh, PhD†; Sanjay K Shukla, PhD**

**Marshfield Clinic Research Foundation, Marshfield, Wisconsin USA*
†A.T. Still University of Health Sciences, Kirksville, Missouri, USA
#Current affiliation: Prevention Genetics, Inc., Marshfield, Wisconsin, USA