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# Staphylococcal superantigen-like genes, *ssl5* and *ssl8*, are positively regulated by Sae and negatively by Agr in the Newman strain

**Madhulatha Pantrangi**[1], **Vineet K. Singh**[2], **Christiane Wolz**[3], and **Sanjay K. Shukla**[1]

[1] Molecular Microbiology Laboratory, Marshfield Clinic Research Foundation, Marshfield, WI, USA

[2] A.T. Still University of Health Sciences, Kirksville, MO, USA

[3] Institut für Medizinsche Mikrobiologie und Hygiene, Tübingen, Germany

## Abstract

Some of the staphylococcal superantigen-like (SSL) proteins SSL5, SSL7, SSL9, and SSL11 act as immunomodulatory proteins in *Staphylococcus aureus*. However, little is known about their regulatory mechanisms. We determined the expression levels of *ssl5* and *ssl8* in seven clinically important *S. aureus* strains and their regulatory mechanisms in the Newman strain, which had the highest *ssl5* and *ssl8* expression. Independent comparisons of *ssl5* or *ssl8* coding and upstream sequences in these strains identified multiple haplotypes that did not correlate with the differential expression of *ssl5* and *ssl8*, suggesting the role of additional regulatory elements. Using knockout mutant strains of known *S. aureus* global regulators such as Agr, Sae, and SigB in the Newman strain, we showed that both *ssl5* and *ssl8* were induced by Sae and repressed by Agr, suggesting that Sae and Agr are the positive and the negative regulators, respectively, of these two *ssl* genes. Moreover, we observed upregulation of *sae* in the *agr* mutant and upregulation of *agr* in the *sae* mutant compared with the isogenic Newman strain, suggesting that the Agr and Sae may be inhibiting each other. The SigB mutation did not affect *ssl5* and *ssl8* expression, but they were downregulated in the *agr*/*sigB* double mutant, indicating that SigB probably acts synergistically with Agr in their upregulation.

### Keywords

*ssl5*; *ssl8*; gene expression; *Staphylococcus aureus*; MRSA; staphylococcal superantigen-like protein

## Introduction

*Staphylococcus aureus* is a significant human pathogen capable of causing a variety of diseases ranging from mild skin and soft tissue infections to bacteremia, pneumonia, endocarditis, and osteomyelitis (Lowy, 1998). The ability of *S. aureus* to cause a wide range of infections is partly due to the expression of a wide array of virulence factors including, but not limited to, cell wall-associated adhesions, clumping factors, exotoxins, and secreted proteins such as staphylococcal superantigen-like (SSL) proteins (Lowy, 1998; Dinges *et al*., 2000; Williams

Correspondence: Sanjay K. Shukla, Molecular Microbiology Laboratory, Marshfield Clinic Research Foundation, 1000 North Oak Avenue-MLR, Marshfield, WI 54449, USA. Tel.: +1 715 389 5363; fax: +1 715 389 3808; shukla.sanjay@mcrf.mfldclin.edu.

Supporting Information

Additional Supporting Information may be found in the online version of this article

*et al*., 2000; Fitzgerald *et al*., 2003). The SSL proteins are encoded by a cluster of 11 *ssl* genes located on *S. aureus* pathogenicity island-2 (Fitzgerald *et al*., 2003). These proteins have limited sequence homology to the enterotoxins and toxic shock syndrome toxin 1 and thus represent a novel family of exotoxin-like proteins (Williams *et al*., 2000). The overall order of *ssl* genes on an *S. aureus* chromosome is conserved, and allelic forms of individual *ssl* genes have been identified in different strains. The sequence homology for individual *ssl* genes ranges from 85% to 100% in different strains. However, 11 *ssl* genes within a strain have sequence homology from 36% to 67%, suggesting possible selective pressures encountered during infection (Kuroda *et al*., 2001; Smyth *et al*., 2007).

Every strain of *S. aureus* examined so far carries a cluster of at least seven of the 11 *ssl* genes, suggesting that they probably have distinct and possibly nonredundant functions (Arcus *et al*., 2002; Fitzgerald *et al*., 2003; Smyth *et al*., 2007). Expression studies of a family of *ssl* genes in COL, an early methicillin-resistant *S. aureus* (MRSA) strain, showed that they are upregulated during the stationary phase like other exotoxin genes (Fitzgerald *et al*., 2003). SSL5 and SSL11 show high structural homology with the chemotaxis inhibitory protein of *S. aureus* and have been shown to interfere with the interaction between P-selectin glycoprotein ligand-1 and P-selectin, suggesting that *S. aureus* uses SSL proteins to prevent neutrophil recruitment towards the site of infection (Bestebroer *et al*., 2007; Chung *et al*., 2007). The same binding site was also found in SSL2, SSL3, SSL4, and SSL6 (Baker *et al*., 2007). SSL7 and SSL9 interact with two separate cell surface ligands of human antigen-presenting cells (monocytes and dendritic cells), leading to internalization by these cells, and may thus play a role in the modulation of host immunity against *S. aureus* (Al-Shangiti *et al*., 2005). In addition, the ability of SSL5, SSL7, SSL9, and SSL11 to impair the protective immune response against *S. aureus* (Al-Shangiti *et al*., 2005; Bestebroer *et al*., 2007; Chung *et al*., 2007) suggests that these proteins could represent potential targets for prophylactic or therapeutic agents to treat invasive staphylococcal diseases (Chung *et al*., 2007). Heme-sensing defective strains of *S. aureus* have shown enhanced expression of *ssl* genes, which was associated with the increased *S. aureus* survival and abscess formation in a host (Torres *et al*., 2007; Langley *et al*., 2009). Despite their well-described role in *S. aureus* pathogenesis, it is not known whether individual SSL proteins are produced in varying amounts in different *S. aureus* clones or multilocus sequence-based sequence types (ST). It is also not known whether genetic polymorphisms in SSL genes influence their expression levels. The aim of this study was to determine the regulatory mechanism of *ssl5* and *ssl8* in clinical strains of *S. aureus* using the Newman as a reference strain.

## Materials and methods

### Bacterial strains

The *S. aureus* wild-type and mutant strains used in this study are listed in Table 1. These strains include three ST8 strains (Newman, FPR3757, and RN6390), two ST5 strains (Mu50 and N315), two ST1 strains (MW2 and MSSA476), and one ST250 strain (COL). Epidemiologically, these strains represent two CA-MRSA strains (FPR3757 and MW2), two nosocomial strains (N315 and MSSA476), two laboratory strains (RN6390 and Newman), one vancomycin intermediate resistance strain (Mu50), and an early MRSA (COL) strain. Because COL lacked *ssl5* and *ssl8* genes, it was used as a negative control in gene expression studies. In addition, the mutant strain of *agr* (accessory gene regulator) (Δ*agr::tetM*, ALC355) (Wolz *et al*., 1996); *sae* (*S. aureus* exoprotein expression) (*sae::*Tn*917*, AS3) (Goerke *et al*., 2001); *sigB* (sigma factor B) (Δ*rsbUVWsigB::erm*(B), IK184) (Kullik *et al*., 1998); and an *agr*/*sigB* double mutant (Δ*agr::tetM*/*sigB::kan*$^{r}$) (VKS104, this study) in the Newman background were used to observe the effect of these regulatory genes on *ssl5* and *ssl8* expression.

### Media and growth conditions

*Staphylococcus aureus* strains were grown either in tryptic soy broth (TSB) or on tryptic soy agar plates (Beckton Dickinson). For broth culture, an overnight shaking culture, grown at 37 °C in TSB, was used to inoculate 50mL of fresh TSB (1: 200 dilutions). Bacterial growth was subsequently monitored by incubating the flask in a shaking incubator and measuring the turbidity of the culture every 30 min at $OD_{600\ nm}$ using a Spectrophotometer (Beckman Coulter Inc., CA) until the culture reached the stationary phase. Cells were collected at the early stationary phase. The MW2, FPR3757, Newman, and MSSA476 reached the early stationary phase ($OD_{600\ nm}$ = 4.5) after 4.5 h, whereas strains RN6390, Mu50, N315, and COL reached the early stationary phase after 5.5 h. The transition phase between the late log phase and the stationary phase was considered as the early stationary phase. None of the Newman mutant strains showed any appreciable growth differences from the Newman wild-type strains (data not shown).

### Construction of the *agr/sigB* knockout mutant

For this study, an *agr*/*sigB* double mutant was generated by transferring the mutation in the *sigB* gene to the *agr* mutant of the Newman strain using a phage transduction procedure as described previously (Singh *et al*., 2003).

### RNA isolation

For gene expression studies, total RNA was isolated at the early stationary phase from all the strains listed in Table 1. Total RNA isolations were performed using a Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's recommendations. The extracted RNA concentration was determined using a 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.

### Relative quantification of *ssl5, ssl8, sae*, and *RNAIII* transcripts by real-time reverse transcriptase (RT)-PCR

We quantified the relative transcript ratio of *ssl5*, *ssl8*, regulatory genes, *sae*, and *agr* (*RNAIII*) against an endogenous control gene, *gmk* (guanylate kinase involved in nucleic acid metabolism), in all the strains mentioned in the Table 1. The extracted RNA samples were treated with RNAse-free DNAse using the Turbo DNA-free™ kit (Ambion, Austin, TX) and confirmed to be DNA free by PCR before cDNA synthesis. cDNA synthesis was performed with 2 μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit following the manufacturer's protocol (Applied Biosystems Inc., Foster City, CA).

From the above reaction mix, ~200 ng of cDNA was mixed with TaqMan Universal PCR Master Mix (2×) (Applied Biosystems Inc.), TaqMan assays containing appropriate PCR primers ($900 nM \mu L^{-1}$) and a 6-FAM dye-labeled MGB probe ($250 nM \mu L^{-1}$). The quantitative real-time PCR was performed in a Light cycler (Roche Diagnostics Corp., Indianapolis, IN). The PCR primers and probes are listed in Table 2. Real-time PCR conditions were as follows: one cycle at 50 °C for 2 min is required for optimal AmpErase UNG activity, one cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min each.

Relative quantifications of *ssl5* and *ssl8* and regulatory gene *agr* (*RNAIII*) and *sae* were determined by measuring against the endogenous control, *gmk*, in the seven clinical and mutant strains (Table 1). Relative quantification was performed using the $2^{-\Delta\Delta C_T}$ calculation according to the manufacturer's guidelines (Roche Diagnostics Corp.). This method compensates factors such as variability in cDNA synthesis and template concentration and calculates transcript ratios (*ssl5*/*gmk*, *ssl8*/*gmk*, *sae*/*gmk*, and *RNAIII*/*gmk*) rather than absolute

values. All of the RT-PCR efficiency was ~2 as required for the reliability of $2^{-\Delta\Delta C_T}$ calculation.

In these experiments, *gmk* was used as a reference gene as its expression levels have been shown to be unchanged under different experimental conditions (Vandecasteele *et al*., 2001; Nieto *et al*., 2009). We confirmed that with equal amounts of RNA in our experiments, the *gmk* transcript levels were the same in the wild-type and the mutant strains. It has been shown that *gmk* works as well an internal control as *gyrA* (Eleaume & Jabbouri, 2004). All RT-PCR results were obtained from two independent cultures.

### Sequencing of *ssl5* and *ssl8*

Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen Inc.) from all the wild-type and the mutant strains mentioned in Table 1. To amplify the *ssl5* and *ssl8* upstream and coding sequences primers were designed to cover the 100 bp upstream promoter region and 705 bp *ssl5* and 699 bp *ssl8* coding regions (Table 3). The amplified products were column purified using the QIAquick PCR Purification Kit (Qiagen Inc.) and sequenced with PCR primers using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems Inc.). Unincorporated dye terminators were removed from the extension products using DyeEx 96 Kit (Qiagen Inc.). Sequences of both strands were analyzed using an ABI Prism 3100 DNA genetic analyzer (Applied Biosystems Inc.). The *ssl5* and *ssl8* sequences obtained were compared against the DNA sequence database in GenBank to confirm their identity.

### Comparative sequence analysis of *ssl5* and *ssl8* coding and the putative promoter region

*ssl5* coding and its 100 bp upstream sequences in the seven clinical strains were compared with each other. A similar comparison was made for *ssl8* alone. The sequence comparison was performed by DNASTAR MEGALIGN program using the CLUSTALW method (LASERGENE, Version 7.2.1, Madison, WI). Allelic forms of the *ssl5* and *ssl8* present in different strains were identified.

### Statistical analysis

Student's *t*-test was used to determine the statistical significance for the gene expression data. *P* values of $<0.05$ were considered to be statistically significant.

## Results

### Relative quantification of *ssl5* and *ssl8* expression in *S. aureus* strains

The expression of *ssl5* and *ssl8* was quantified at the early stationary phase in all the strains listed in Table 1. As expected, the negative control strain, COL, did not show *ssl5* or *ssl8* expression as it lacked these genes. Both *ssl5* and *ssl8* had the highest expression in the Newman strain, whereas MW2 and Mu50 strains had the lowest expression, respectively. Both *ssl5* and *ssl8* expression levels varied in strains within an ST and also when compared among strains with different STs (Fig. 1). The ST8 strains, RN6390 and FPR3757, showed *ssl5* levels comparable to each other; however, they had fourfold less expression compared with the Newman strain. In the case of ST1 strains, MSSA476 showed fivefold higher *ssl5* expression compared with the MW2 strain. However, MSSA476 and MW2 strains showed 1.5- and 7-fold lower *ssl5* expression, respectively, in comparison with the Newman strain. The ST5 strains, Mu50 and N315, showed similar *ssl5* expression levels, but showed three- and four-fold less expression, respectively, when compared with the Newman strain (Fig. 1).

The *ssl8* expressions were relatively similar in RN6390 and FPR3757. However, its expression was 12- and 20-fold lower in RN6390 and FPR3757, respectively, compared with the Newman strain. The MW2 strain showed threefold higher *ssl8* levels compared with MSSA476;

however, these strains showed 13- and 40-fold less *ssl8* expression, respectively, compared with the Newman strain. In N315 and Mu50, the *ssl8* levels were similar to each other, but in a negligible amount when compared with the Newman strain (Fig. 1). When the expression levels of *ssl5* and *ssl8* were compared, they were found to be similar in RN6390 and FPR3757, but *ssl8* expression was fourfold higher in the Newman strain compared with *ssl5*. Interestingly, MW2 had twofold higher *ssl8* levels compared with *ssl5*, whereas MSSA476 showed sevenfold higher *ssl5* levels compared with s*sl8* levels. In contrast, Mu50 and N315 showed 17- and 10-fold higher *ssl5* levels, respectively, compared with their *ssl8* expression levels (Fig. 1). The differential expression of both *ssl5* and *ssl8* in different strains prompted us to see whether different haplotypes of *ssl5* and *ssl8* are present in these strains and whether they correlated with their differential expression.

## Allelic differences within *ssl5* and *ssl8* in the clinical strains tested

We sequenced *ssl5*, *ssl8* and their 100 bp upstream regions from the seven clinical strains and various Newman mutant strains used in this study. Because the Newman strain had the highest expression of both *ssl5* and *ssl8* compared with the other clinical strains tested, the *ssl5*, *ssl8* and their 100 bp upstream sequences obtained were compared with the respective genes of this strain to determine any allelic differences. Based on the respective comparison of *ssl5* and *ssl8* coding sequences of the seven strains tested (Table 1), three haplotypes emerged. Haplotype A included Newman, FPR3757, and RN6390 strains; haplotype B included MW2 and MSSA476 strains; and haplotype C included Mu50 and N315 strains (Figs 2a and 3a). For the *ssl5* or *ssl8* upstream sequence comparative analysis, three allelic forms were identified for each one. For both *ssl5* and *ssl8*, allelic type A included the same three strains: Newman, FPR3757, and RN6390. However, for *ssl5*, allelic type B included MW2, MSSA476, and N315, whereas allelic type C included Mu50 (Fig. 2b). For *ssl8*, allelic type B included MW2, Mu50, and N315, whereas allelic type C included MSSA476 (Fig. 3b).

The *ssl5* and *ssl8* coding and promoter sequences showed several single nucleotide polymorphisms (SNPs) (Figs 2a, b and 3a, b). These SNPs and the corresponding amino acid change in the coding region were described in Supporting Information, Tables S1 and S2. There was no correlation between haplotypes or allelic types relative to *ssl5* or *ssl8* expression. The differential expressions of *ssl5* and *ssl8* within a haplotype with identical upstream sequences in strains such as Newman, RN6390, and FPR3757 suggested that their expression was influenced by additional factors (Fig. 1).

## Effects of staphylococcal global regulators on *ssl5* and *ssl8* expression

Using Newman as the model strain because of its highest expression of *ssl5* and *ssl8*, we determined the role of known regulatory elements, Agr, Sae, and SigB, in their expression. Relative expressions of *ssl5* and *ssl8* were compared in the wild-type Newman strains with isogenic mutant strains of *agr*, *sae*, *sigB* and the *agr*/*sigB* double mutant to determine their role in *ssl5* and *ssl8* regulation. The mutant strains did not show any growth difference compared with the wildtype Newman strain (data not shown).

Both *ssl5* and *ssl8* expression showed upregulation in the *agr* mutant and downregulation in the *sae* mutant compared with the wild-type Newman strain (Fig. 4), suggesting that the Agr system is a negative regulator and Sae is a positive regulator for the expression of *ssl5* and *ssl8* genes. In order to clarify the role of the Agr, we also measured the *RNAIII* transcript level, which has been shown to regulate the expression of many exoproteins in *S. aureus* (Peng *et al*., 1988;Novick *et al*., 1993). In the seven strains tested, the relative *RNAIII* transcript levels varied and ranged from $1.5 \times 10^{-4}$ to 243-folds with reference to *gmk* transcript levels (Fig. 1). However, no correlation between *RNAIII* and *ssl5* or *RNAIII* and *ssl8* expression was observed in any of the wild type reference strains tested (Fig. 1). We checked the expression

of *sae* in all the reference strains and found that *sae* expression was 7–36-fold higher in the Newman strain compared with the other six strains used in this study.

In the *sae* mutant, the level of *RNAIII* was higher (3.5-fold), but the transcript levels of both *ssl5* and *ssl8* were lower by 4- and 28-fold, respectively, compared with their levels in the wild-type Newman (Fig. 4). In the *agr* mutant, transcript levels of *sae*, *ssl5*, and *ssl8* were higher by 2.5-, 2-, and 3-fold, respectively, compared with their respective levels in the wild-type Newman. There was no change in the expression of either *ssl5* or *ssl8* in the Newman strain (Fig. 4) that had a *sigB* mutation. However, in a *sigB*/*agr* double mutant of Newman that expressed 56-fold less *sae*, expressions of *ssl5* and *ssl8* were also repressed by 3- and 20-fold, respectively, relative to the wild-type Newman strain. These data collectively suggest SaeR/S to be a major positive regulator and Agr to be a negative regulator of *ssl5* and *ssl8* gene expression in Newman.

## Discussion

Staphylococcal extracellular virulence factors are accessory gene products that contribute significantly to *S. aureus* pathogenicity (Lowy, 1998; Dinges *et al*., 2000). Their production is often dependent on quorum sensing (Geisinger *et al*., 2008) and controlled by a network of global regulators including the two-component regulatory system, Agr and Sae, which act at the transcriptional level (Novick & Jiang, 2003). Sae induces the expression of several virulence factors such as coagulase (Coa), α-hemolysin (Hla), β-hemolysin (Hlb), extracellular adherence protein (Eap), extracellular matrix binding protein (Emp), protein A, and fibronectin-binding proteins (FnbA and FnbB) (Goerke *et al*., 2001; Harraghy *et al*., 2005). In contrast, the Agr inhibits the expression of *coa*, *fnbB*, and *fnbA*, indicating that Agr might act as an antagonist of Sae (Wolz *et al*., 1996). Others have reported that *sae* is downstream from *agr* in the regulatory pathway or perhaps epistatic (Giraudo *et al*., 2003; Novick & Jiang, 2003), suggesting that the *sae* transcription could be influenced by Agr in some strains, but acts independent of Agr in other strains (Ross & Novick, 2001).

In the present study, we describe the expression pattern of *ssl5* and *ssl8* in the early stationary phase in several *S. aureus* strains belonging to different clones. It appears that the regulation of *ssl5* and *ssl8* expression in *S. aureus* is strain specific as they varied even within an ST and gene haplotype (Fig. 1). *Staphylococcus aureus* is known to show a differential expression of genes implicated in virulence. Harraghy *et al*. (2005) observed marked differences in the expression of staphylococcal adhesins, *eap* and *emp* between Newman and NCTC8325 derivative strains, SH1000 (8325-4 $rsbU^+$) and 8325-4 ($rsbU^-$). Our data show that the *ssl5* and *ssl8* expression is downregulated in the *sae* mutant strain and upregulated in the *agr* mutant strain, suggesting that Sae and Agr are possible inducers and repressors, respectively, for *ssl5* and *ssl8* in the Newman strain (Fig. 4). Indeed, downregulation of several proteins including SSL7 and SSL11 has been observed in a Newman *sae* mutant strain (Rogasch *et al*., 2006). The Newman strain is characterized by unusually high *sae* levels, which have been confirmed in this study as well. The high *sae* expression in this strain can be attributed to a point mutation in the sensor histidine kinase of the SaeR/S two-component regulatory system (Steinhuber *et al*., 2003;Geiger *et al*., 2008). Proteomics and microarray analyses have revealed that most of the genes influenced by Sae are involved in bacterial adhesion, immune evasion, immune modulation, or toxicity (Foster, 2005;Liang *et al*., 2006;Rogasch *et al*., 2006). More importantly, it has been shown that *sae* is essential for virulence gene expression *in vivo* (Goerke *et al*., 2001). It was interesting to observe the suppressive effect of Agr on *ssl5* and *ssl8* expression, suggesting that Agr does not always act as a positive regulator for virulence gene expression in *S. aureus*, and inhibiting the Agr function to reduce virulence could have other consequences (Otto, 2001). Loss of Agr increases the bacterial colonization, biofilm formation, and attachment to polystyrene, suggesting that the *agr* mutant strain may have a

greater capacity to cause chronic infections than *agr*-positive strains (McNamara & Bayer, 2005).

We speculated that the lack of Agr could have caused the enhanced expression of some proteins that aid in the upregulation of *ssl5* and *ssl8*. Surprisingly, we found that the *agr* mutation caused increased *sae* transcript levels and vice versa, which indicated that the *sae* and *agr* could have an inhibitory effect on each other, and repression of *ssl5* and *ssl8* genes by Agr is dependent on Sae in the Newman background. This observation is in contrast to a previous report suggesting that the *sae* does not effect the transcription of other regulatory genes where they have observed strong signals for *RNAIII* in both RN6734, a Φ13 lysogen of RN6390, and its *sae* mutant strain, RN9808 (Novick & Jiang, 2003). However, Voyich *et al*. (2009) reported that *ssl11* and the *agr* operon in a *saeR*/*S* mutant of MW2 strain is downregulated by ~16- and 2-fold, respectively, at the early stationary growth phase. In concordance with our data, Liang *et al*. (2006) showed, by RT-PCR analysis, that *agrA* mRNA levels were significantly upregulated in the *saeS* null mutant compared with its wild-type strain, WCUH29, a virulent clinical isolate. Taken together, these data suggest that the influence of *saeR*/*S* on the transcriptional regulation of virulence genes is probably dependent on multiple factors including the genomic background of the strain studied (Liang *et al*., 2006; Rogasch *et al*., 2006).

Interestingly, in the *agr*/*sigB* double mutant, the expressions of *ssl5*, *ssl8*, and *sae* was downregulated (Fig. 2). However, in the *agr* mutant strain, these genes were upregulated, whereas the expression of either *ssl5* or *ssl8* did not change in a Newman *sigB* mutant. This suggests that SigB probably acts synergistically with Agr, but not alone, to upregulate *ssl5* and *ssl8*. This could very well be mediated by *sae* specifically in the Newman strain. An analogous phenomenon such as enhanced repression of exotoxin-encoding genes in double mutants of regulatory genes in *S. aureus* is not uncommon. For example, *sar* and *agr* double mutants are less virulent compared with the *agr* single mutant (Booth *et al*., 1997). Differences in the transcript levels of regulatory genes (*agr*, *sarA*, *sigB*, and *saeR*/*S*) have been reported between COL and Newman strains that correlate well with the expression of virulence-associated genes (Rogasch *et al*., 2006).

In summary, *ssl5* and *ssl8* expression in *S. aureus* clinical isolates is strain dependent and not influenced by differences in their alleles. They are positively regulated by Sae and negatively by Agr in the Newman strain. Furthermore, the *ssl5* and *ssl8* repression by Agr is probably achieved by the downregulation of Sae in the Newman strain. This is the first report of a negative regulation of an *ssl* gene by Agr. This study also highlights the potential challenges in managing infections due to *S. aureus* strains, which could potentially produce varying amounts of SSLs. Understanding the intricacy of global regulatory genes and their mode of regulation in different genetic backgrounds would provide an important insight into the molecular mechanisms of staphylococcal virulence. This may perhaps reveal specific targets, which would enable therapeutic intervention in *S. aureus* infections.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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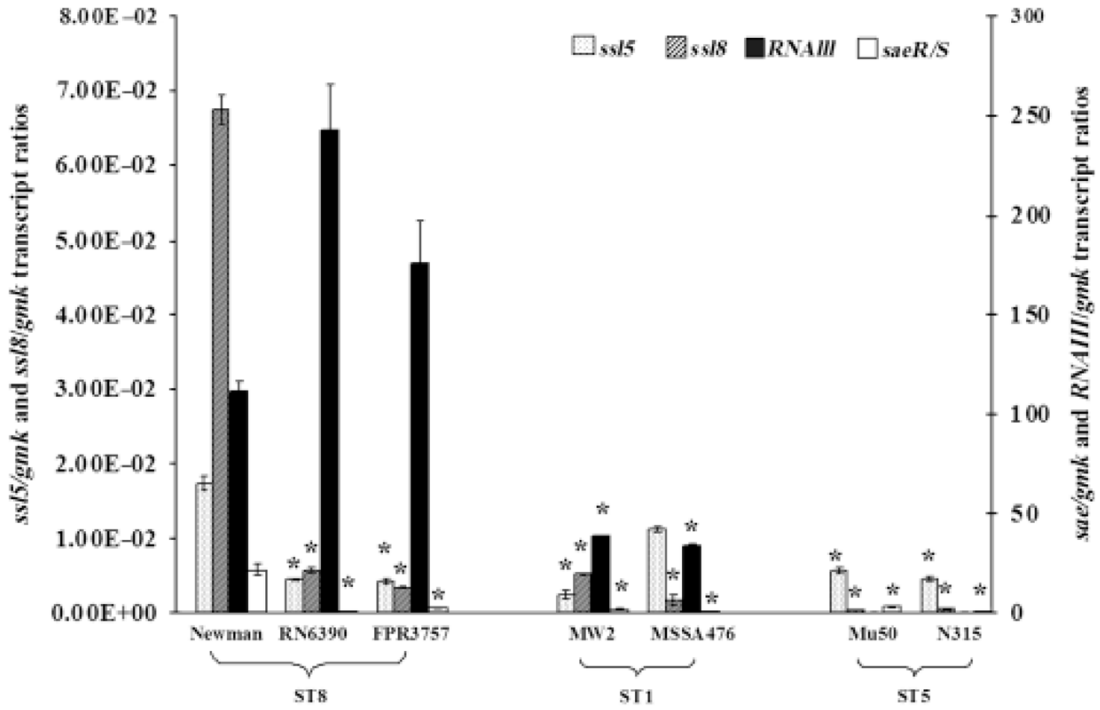


**Fig. 1.**

Transcript ratios of *ssl5*/*gmk*, *ssl8*/*gmk*, *RNAIII*/*gmk*, and *sae*/*gmk* quantified at the early stationary phase of seven strains: RN6390, FPR3757, Newman, MW2, MSSA476, Mu50, and N315. The dotted and the hatched bars on the primary axis show *ssl5*/*gmk* and *ssl8*/*gmk* transcript ratios, respectively. The closed and open bars are relative to the secondary axis and show *RNAIII*/*gmk* and *sae*/*gmk* transcript ratios. Data represented here show the mean values of two independent measurements. An asterisk (*) denotes that the values are statistically significant (i.e. $P<0.05$, by Student's *t*-test) compared with the Newman strain.

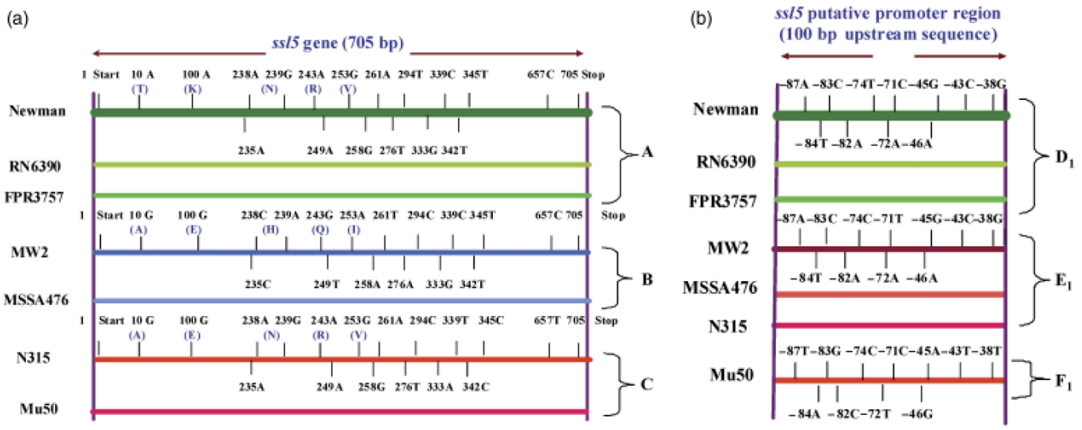


**Fig. 2.**

(a) Schematic comparison of the *ssl5* gene sequences found in seven *Staphylococcus aureus* strains mentioned in Fig. 1. Different-colored horizontal lines represent different haplotypes (A, B, and C) as indicated on the right side. Only differences in the SNPs and the corresponding amino acid (in parentheses) with respect to the Newman strain are shown. The thick green horizontal line at the top represents the *ssl5* sequence from the Newman strain. Short vertical lines above and below the horizontal lines indicate the relative positions of SNPs on the gene. The purple long vertical lines at two ends indicate the 5′ and 3′ ends of *ssl5*. T, threonine; K, lysine; N, asparagine; R, arginine; V, valine; A, alanine; E, glutamic acid; H, histidine; Q, glutamine; and I, isoleucine. (b) The SNPs in the 100 bp upstream sequences of the *ssl5* putative promoter region from the strains mentioned in Fig. 1. The SNPs among seven strains are shown with reference to the Newman strain. The thick green horizontal line at the top represents the *ssl5* putative promoter sequence from the Newman strain. Short vertical lines above and below the horizontal lines indicate the relative positions of SNPs. The right vertical line indicates the beginning of the upstream sequence. Three allelic subtypes ($D_1$, $E_1$, and $F_1$) are indicated on the right side and strain names on the left side.

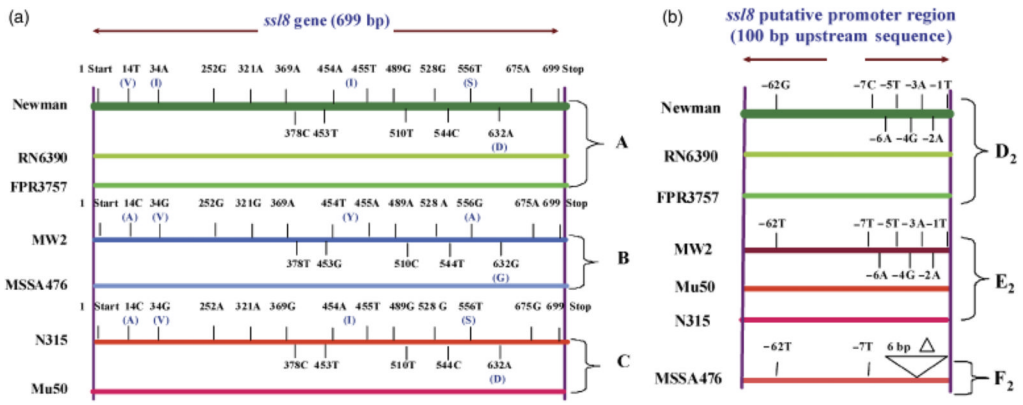


**Fig. 3.**

(a) Schematic diagram of the *ssl8* gene sequences from the seven *Staphylococcus aureus* strains mentioned in Fig. 1. Different-colored horizontal lines represent different *S. aureus* strains as indicated on the left side. Haplotype groups (A, B, and C) are indicated on the right side. The SNPs and the corresponding amino acid (in parentheses) changes with respect to the Newman strain are shown. The thick green horizontal line at the top represents the *ssl8* sequence from the Newman strain. Short vertical lines above and below the horizontal lines indicate the relative positions of SNPs on the gene. The purple long vertical lines at two ends indicate the 5′ and 3′ ends of *ssl8*. V, valine; I, isoleucine; S, serine; A, alanine; Y, tyrosine; G, glycine; and D, aspartic acid. (b) The SNPs in the 100 bp upstream sequences of the *ssl8* putative promoter sequence from strains mentioned in Fig. 1. The deletion and SNPs among seven strains with reference to the Newman strain are shown. The thick green horizontal line at the top represents the *ssl8* putative promoter sequence from the Newman strain. Short vertical lines above and below the horizontal lines indicate the relative positions of SNPs. The right vertical line indicates the beginning of the upstream sequence. Symbol ‘Δ’ denotes the deletion. Three allelic subtypes ($D_2$, $E_2$, and $F_2$) are indicated on the right side and strain names on the left side.

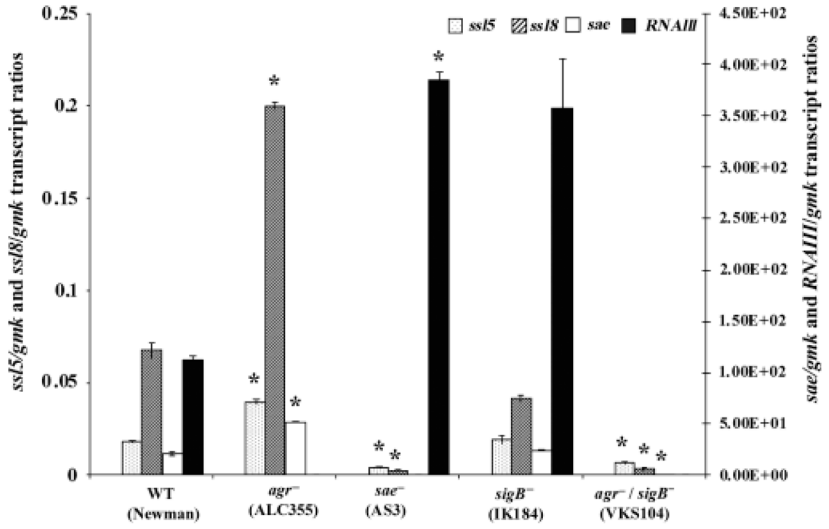


**Fig. 4.**

Transcript ratios of *ssl5*/*gmk*, *ssl8*/*gmk*, *sae*/*gmk*, and *RNAIII*/*gmk* quantified at the early stationary phase in the Newman strain and its isogenic mutants: ALC355 (*agr*$^{-}$), AS3 (*sae*$^{-}$), IK184 (*sigB*$^{-}$), and VKS104 (*agr*$^{-}$/*sigB*$^{-}$). The dotted and the hatched bars on the primary axis show *ssl5*/*gmk* and *ssl8*/*gmk* transcript ratios, respectively. Open and closed bars with respect to the secondary axis show *sae*/*gmk* and *RNAIII*/*gmk* transcript ratios, respectively. Data here represent the mean values of two independent measurements. An asterisk (*) shows that the values are statistically significant (i.e. $P<0.05$, by Student's *t*-test) compared with the wild-type strain Newman.

## Table 1

*Staphylococcus aureus* strains used in this study

| ***S. aureus* strains** | **Source, genotype, and phenotype description** | **References** |
|---|---|---|
| *Wild-type strains* | | |
| MW2 (NRS123) | CA-MRSA; *agr* group III; *spa* type t128; ST1; $PVL^+$ | Baba *et al*. (2002) |
| FPR3757 (NRS384) | CA-MRSA; *agr* group I; *spa* type t008; ST8; $PVL^+$ | Diep *et al*. (2008) |
| COL (NRS100) | An early MRSA; ST250 | Gill *et al*. (2005) |
| NCTC8325/RN6390 (NRS147) | Laboratory strain; *agr* group I; ST8 | Peng *et al*. (1988) |
| Newman | A clinical isolate, routinely used as a laboratory strain; *spa* type t008 | Baba *et al*. (2008) |
| MSSA476 | Nosocomial strain; ST1 | Holden *et al*. (2004) |
| N315 | Nosocomial strain; ST5 | Kuroda *et al*. (2001) |
| Mu50 | Vancomycin-intermediate resistant *Staphylococcus aureus*; ST5 | Hiramatsu *et al*. (1997) |
| *Mutant strains* | | |
| ALC355 | Newman Δ*agr::tetM* | Wolz *et al*. (1996) |
| AS3 | Newman *sae*::Tn*917* | Goerke *et al*. (2001) |
| IK184 | Newman Δ*rsbUVWsigB*; $Em^r$ | Kullik *et al*. (1998) |
| VKS104 | Newman, Δ*agr::tetM*/*sigB::kan*$^r$ | This study |

CA-MRSA, community-associated methicillin-resistant *S. aureus*.

**Table 2**

Primers and probes used in this study

| Genes | Primer sequence (50′ → 30′) | Probe sequence (50′ FAM → 30′ NFQ) | References |
|---|---|---|---|
| *ssl5* | | | |
| Forward | GGTGGTGTCACTAAGAAAAATCAAGAC | ACGCACCAAGATTTC | This study |
| Reverse | CAATACCGTCACCTTCATCTCTCTT | | This study |
| *ssl8* | | | |
| Forward | GTTCCAAGTGTTTTTATTGGGAAAAGATGA | ACACATGGTTTAGATGTCTTTG | This study |
| Reverse | GTTACACCACTAACACTAAATATTCTTCCATCTA | | This study |
| *gmk* | | | |
| Forward | ACTAGGGATGCGTTTGAAGCTTTAA | AAAGATGACCAATTTATAGAATATG | This study |
| Reverse | CTGGTGTACCATAATAGTTGCCTACAT | | This study |
| *sae* | | | |
| Forward | CAACTGTCGTTTGATGAATTAACACTTATTAACT | ATGGTCACGAAGTCCC | This study |
| Reverse | CCACAATAACTCAAATTCCTTAATACGCAT | | This study |
| *RNAIII* | | | |
| Forward | TCCATTTTACTAAGTCACCGATTGT | ATCTTGTGCCATTGAAATCACTCCTTCCTT | Loughman *et al.* (2009) |
| Reverse | TGTGATGGAAAATAGTTGATGAGTTGT | | Loughman *et al.* (2009) |

## Table 3

Sequencing primers used in this study

| Genes | Target strains | Primer sequence (5′ → 3′) | References |
|---|---|---|---|
| *ssl5*-F | Mu50 | TCCTCAAATGTGCCAAGTGT | This study |
| *ssl5*-F | Newman, RN6390, FPR3757, MW2, MSSA476, and N315 | CAGTTTTATTTAACGAACATTATAGATTCC | This study |
| *ssl5*-R | Newman, RN6390, FPR3757, MW2, MSSA476, Mu50, and N315 | CAACTTATGTTGCCTAACTCCTC | This study |
| *ssl8*-F | Newman, RN6390, and FPR3757 | TGAAAGTGATGCCCATTGAA | This study |
| *ssl8*-F | MW2, MSSA476, Mu50, and N315 | CTTCTGAAAGTGATGTCCATTGAA | This study |
| *ssl8*-R | Newman, RN6390, FPR3757, MW2, MSSA476, Mu50, and N315 | ACATGGGATTATTAAACCGCTTC | This study |