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TOPIC HIGHLIGHT

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Transcribing virulence in *Staphylococcus aureus*

Jennifer M Junecko, Agnieszka K Zielinska, Lara N Mrak, Douglas C Ryan, Justin W Graham, Mark S Smeltzer, Chia Y Lee

Jennifer M Junecko, Agnieszka K Zielinska, Lara N Mrak, Douglas C Ryan, Justin W Graham, Mark S Smeltzer, Chia Y Lee, Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, AR 72205, United States

Author contributions: Junecko JM, Zielinska AK, Ryan DC and Graham JW prepared the initial draft of the paper; Junecko JM and Mrak LN contributed to critical review, editing and revision; Zielinska AK provided illustrations; Smeltzer MS and Lee CY edited the final draft.

Correspondence to: Chia Y Lee, PhD, Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, 4301 W. Markham St. Mail Slot 511, Little Rock, AR 72205, United States. clee2@uams.edu

Telephone: +1-501-5267687 Fax: +1-501-8685359
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Abstract

Staphylococcus aureus (*S. aureus*) is an important human pathogen capable of causing a diverse range of infections. Once regarded as an opportunistic pathogen causing primarily nosocomial infections, recent years have seen the emergence of *S. aureus* strains capable of causing serious infection even in otherwise healthy human hosts. There has been much debate about whether this transition is a function of unique genotypic characteristics or differences in the expression of conserved virulence factors, but irrespective of this debate it is clear that the ability of *S. aureus* to cause infection in all of its diverse forms is heavily influenced by its ability to modulate gene expression in response to changing conditions within the human host. Indeed, the *S. aureus* genome encodes more than 100 transcriptional regulators that modulate the production of virulence factors either directly *via* interactions with *cis* elements associated with genes encoding virulence factors or indirectly through their complex interactions with each other. The goal of this review is to summarize recent work describing these regulators and their contribution to defining *S. aureus* as a human pathogen.

Key words: *Staphylococcus aureus*; Regulatory elements; DNA-binding proteins; Two-component systems; Virulence factors

Peer reviewer: Dr. Janne Kudsk Klitgaard, Institute of Clinical Microbiology, University of Southern Denmark, Campusvej 55, Odense M 5230, Denmark

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a rapidly evolving human pathogen that is a leading cause of both chronic, biofilm-associated infections and acute, life-threatening toxemias. Its ability to cause these infections is dependent on its ability to coordinate the production of a myriad of virulence factors. These include exopolysaccharides, surface-associated protein adhesins, immune modulators, and extracellular proteins including a plethora of toxins. *S. aureus* employs an equally remarkable array of regulatory elements to coordinate the production of these virulence factors. These elements include (1) small, non-coding RNAs[1]; (2) alternative sigma factors (σ^B, σ^H, and σ^S) responsive to various stress conditions[2]; and (3) *trans*-acting transcriptional regulators. It is the latter two groups that is the focus of this review. Indeed, one of the first reports of a full *S. aureus* genome sequence identified 124

open-reading frames likely to encode transcriptional regulators, 89 of which had not been previously identified[3]. Generally speaking, these are DNA-binding proteins, although some have also been shown to modulate virulence phenotypes *via* direct interactions with mRNA[4]. In general, these factors, some upon activation, are capable of binding a specific sequence associated with their target genes and thereby either enhance or inhibit transcription, although in many cases it has proven difficult to identify a definitive recognition site. Many of these targets are themselves regulatory factors, thus creating a complex network of virulence gene expression.

Because DNA-binding proteins are located in the cytoplasm, it is imperative to have a mechanism of sensing the external environment and translating that information into an intracellular change in gene expression. As with other pathogens, this is often accomplished *via* two-component systems. In some cases, the activating signals for these systems are known, but in most cases they have not been defined. *S. aureus* also produces many transcriptional regulators that are not associated with a recognized two-component system, and the activating signals for most of these also remain undefined. Nevertheless, an important role in virulence has been established for many of these regulators, and summarizing these is the focus of this review.

TWO-COMPONENT SYSTEMS

Bacterial two-component systems create a communication bridge to the external environment, allowing the cell to translate an external stimulus into an intracellular change in gene expression. The defining components are a membrane-associated sensor histidine kinase (HK) and a cytoplasmic response regulator (RR). The genes encoding these components are often arranged in an operon, and therefore co-transcribed, with other genes involved in the same signaling pathway. After activation from an external signal, the HK typically dimerizes and trans-autophosphorylates[5]. This leads to phosphorylation of the RR, characteristically at a conserved aspartic acid residue. Phosphorylation of the RR induces a conformational change allowing it to bind DNA at a specific consensus sequence in a manner that alters transcription of the target gene. Based on homology with recognized sensors and response regulators, *S. aureus* has at least 16 two-component systems[6]. In addition to their role in pathogenesis, at least three of these systems have been shown to modulate resistance to antibacterial agents[7], thus further emphasizing their important role in pathogenesis of *S. aureus* infection.

AgrAC

The most definitively characterized two-component system in *S. aureus* is the accessory gene regulator (*agr*), which was first identified as a transposon-insertion mutant with a reduced capacity to produce multiple exotoxins[8]. AgrC is the sensor kinase, and it is responsive to the accumulation of an extracellular auto-inducing peptide (AIP) that is encoded by *agrD* and processed for export by AgrB. Induction occurs *in vitro* as cultures enter the post-exponential growth phase as the AIP accumulates, thus making *agr* a prototype quorum-sensing regulatory system. This peptide recognition is a unique feature of Gram-positive quorum-sensing systems, as Gram-negative organisms sense small molecules, typically homoserine lactones[9], rather than peptides. While *agr* itself is highly conserved, variation in the AIP and its AgrC receptor define interference groups, with the AIP of each group inducing the expression upon interaction with its cognate receptor but inhibiting induction upon interaction with the receptor from each of the other groups[10].

After induction by AIP, AgrC autophosphorylates[11] and then phosphorylates AgrA, which is the cytoplasmic response regulator that until recently was thought to bind and activate only the *agr*-associated P2 and P3 promoters. Induction of the P2 promoter leads to increased transcription of the *agr* operon (*agrABCD*), resulting in a positive feedback loop, while induction of the P3 promoter results in increased transcription of RNAIII, with the latter being a primary downstream effector of the *agr* system[12].

AgrA was also recently shown to bind the promoter region of the gene clusters encoding phenol-soluble modulins (PSMs)[13]. PSMs are small toxins that lyse human neutrophils[14], a key host defense against staphylococcal infection. While PSMs are found in virtually all *S. aureus* isolates, the levels in which they are produced vary widely among different strains due to differences in the level of *agr* expression. This has been correlated with increased virulence in several animal models of *S. aureus* infection, although not necessarily owing to the increased production of PSMs alone[15-17].

RNAIII is the effector molecule of the *agr* regulatory system. Although RNAIII includes the gene encoding delta-toxin, which is itself a PSM[18], its primary contribution to virulence is regulatory. It is a stable RNA characterized by 14 stem-loop structures and two long helices separating two independent domains[19], and it is these stem-loops that are responsible for its regulatory effects[1]. RNAIII production is induced by the binding of phosphorylated AgrA to the P3 promoter, thus accounting for its increased production *in vitro* as cultures enter the post-exponential growth phase and AIP accumulates to a critical threshold. In general, induction results in reduced production of surface-associated proteins and enhanced production of exotoxins[20].

The phenotype of an RNAIII mutant is characterized by major changes at the transcriptional level. However, RNAIII itself functions primarily at a post-transcriptional level to affect accessory transcription factors leading to changes in virulence gene expression. For instance, transcription of the gene encoding staphylococcal protein A (*spa*) is increased in the absence of RNAIII, but this is due to the fact that RNAIII normally represses production of other transcription factors (e.g., SarT, Rot,

and ultimately SarS) that would otherwise promote *spa* transcription[21]. Thus, in the absence of RNAIII, this repression does not occur, which results in the continued high level expression of *spa*. Additionally, RNAIII binds *spa* mRNA in a manner that both limits translation and promotes RNase III-mediated degradation[22].

This latter mechanism also plays a primary role in the RNAIII-mediated induction of toxin production[20]. This occurs *via* both direct and indirect pathways. For instance, in the case of *hla*, which encodes α toxin, the *hla* transcript forms a stem-loop structure that sequesters the Shine-Delgarno sequence, thus limiting translation. RNAIII overcomes this limitation by binding the *hla* transcript and relieving this stem-loop structure[23]. The translation of *hla* is thus upregulated in the presence of RNAIII *via* a direct interaction between RNAIII and *hla* mRNA. In other cases, the regulatory functions are mediated indirectly *via* the interaction between RNAIII and the *rot* transcript. Specifically, the regulatory functions of Rot (repressor of toxins) and Agr are antagonistic, with RNAIII limiting the production of Rot by binding to the Shine-Dalgarno sequence of the *rot* transcript and, as with the *spa* transcript, both inhibiting translation and targeting the existing transcript for degradation by RNaseIII[1]. In addition to its regulation of the genes encoding individual virulence factors, RNAIII also modulates the expression of other two-component systems including ArlRS, SaeRS and SrrAB, but the mechanism by which this occurs is not known[24-26].

Taken together, these results imply that *agr* plays a central role in *S. aureus* regulatory circuits. This is also reflected in the observation that mutation of *agr* has been consistently associated with a reduced capacity to cause infection[27]. Indeed, a primary determinant of the hypervirulence of isolates of the USA300 clonal lineage is their high level expression of *agr* and consequent high level production of critical exotoxins including α toxin and PSMs[27]. At the same time, this does not mean that *agr* expression is critical in all forms of *S. aureus* infection. One specific phenotype that may be particularly important in this regard is biofilm formation, with the high-level expression of *agr* generally being associated with a reduced capacity to form a biofilm[28-30]. It has been proposed that induction of *agr* expression may be important in promoting dispersal of *S. aureus* cells from an established biofilm, perhaps by inducing the production of extracellular proteases and/or nucleases[31]. This suggests that the expression of *agr* needs to be carefully controlled in the cells during biofilm development. However, several reports have documented the isolation of *agr* mutants directly from patients suffering from *S. aureus* infection[32,33], and it has even been suggested that *agr* dysfunction may be adaptive for survival within an infected host[34]. In fact, this is one specific context in which *agr* mutants have been shown to preferentially accumulate[34], perhaps owing to both the negative impact of *agr* on biofilm formation and the fact that its expression is metabolically expensive[35].

SaeRS

The *saeRS* two-component system was first identified as a transposon-insertion mutant deficient in exoprotein production[36]. *saePQRS* is transcribed as a 4-gene operon (*saePQRS*), with SaeS and SaeR being the sensor and response regulator respectively. A definitive role for SaeP and SaeQ has not yet been determined, although they may be involved in stabilization of SaeS in the membrane and/or modulating its return to the dephosphorylated state[37]. Once phosphorylated, SaeR binds to a specific target sequence ($GTTAAN_6GTTAA$) to activate transcription of *saePQRS* itself[38]. This is very similar, although not identical, to the AT-rich consensus binding site identified by Nygaard *et al*[39] based on alignments with additional SaeR-regulated target genes[39].

Several studies have demonstrated that *sae* also modulates the production of virulence factors other than toxins including surface proteins and capsule biosynthesis components[38,40-43]. Several experimental observations suggest that *saePQRS* is downstream of *agr*, as well as other regulatory loci. Transcription of *saePQRS* is activated by *agr* but is repressed by SigB, while SaeRS does not seem to affect transcription of *agr*, *sigB* or *sarA,* suggesting that SaeRS acts as an important downstream regulator within the *S. aureus* global regulatory network[36,42,44,45]. Genetic experiments on exoprotein production also suggest that *saePQRS* is downstream of and epistatic to *agr*[42]. Furthermore, inactivation of either *agr* or *sae* had a comparable impact on the virulence of a USA300 isolate in a murine pneumonia model[46]. However, while inactivation of *agr* or *sae* results in reduced production of extracellular proteins, the exoprotein profiles of the two mutants are not identical[36,47]. It is also clear from several studies that the two regulons are not equivalent[39,41,43]. For instance, inactivation of *sae* results in decreased transcription of the *fnbA* and *fnbB* genes[39,41], both of which encode fibronectin-binding proteins, while inactivation of *agr* has the opposite effect[48]. Thus, while *sae* seems to function downstream of *agr*, it is also capable of regulating its target genes independent of *agr*.

One of the most commonly studied strains of *S. aureus* is Newman, which has a naturally occurring point mutation in *saeS* resulting in substitution of a leucine with a proline (L18P). This results in increased kinase activity leading to constitutive activation of SaeR and increased transcription of the *saePQRS* genes[49]. However, only certain target genes within the SaeRS regulon are differentially regulated in Newman due to the polymorphism of SaeS. Class I target genes are sensitive to the $SaeS^P$ allele and Class II genes are not. Although the mechanistic basis for this difference is not clear, it does not appear to be due to a gene dosage effect[41]. When $SaeS^L$ is cloned into wild-type Newman, it is dominant over $SaeS^P$, suggesting instability of the system upon over-production, perhaps due to SaeS phosphatase rather than kinase activity[41].

Inactivation of *sae* is associated with increased transcription of several genes encoding extracellular proteases and increased accumulation of the corresponding

proteases themselves[50], and this may well have an indirect effect on other virulence phenotypes of *S. aureus*. For instance, Newman is one of the few strains in which inactivation of the staphylococcal accessory regulator (*sarA*) does not result in an α toxin-deficient phenotype, and it was recently demonstrated that this is due to the hyperactivity of $SaeS^P$ leading to the reduced production of extracellular proteases, and consequent reduced degradation of the toxin, rather than transcriptional changes associated with *hla*[51].

The environmental cues modulating SaeRS activity have not been clearly defined but are associated with stress conditions including high salt, low pH, and subinhibitory concentrations of antibiotic[44]. Because SaeRS is induced by hydrogen peroxide and α-defensins, and because many toxins are SaeRS-regulated, it has been hypothesized that this system could promote escape from polymorphonuclear leukocytes after phagocytosis[44]. Indeed, it has been demonstrated that an *saeRS* mutant strain has an impaired ability to survive in human neutrophils after phagocytosis[43].

ArlRS

Fournier *et al*[52] used transposon mutagenesis to identify genes involved in the regulation of the multidrug efflux pump NorA and identified *arlS*, inactivation of which resulted in increased resistance to quinolones. ArlS is the sensor and ArlR is the response regulator of this two-component system. A subsequent study confirmed that ArlRS also modulates the production of exoproteins, but in this case the phenotype was opposite to that of an *agr* mutant, with an *arlRS* mutant exhibiting increased production of multiple exoproteins[52]. Additionally, an *agr*/*arl* double mutant exhibited an exoprotein phenotype comparable to the isogenic *arl* mutant, suggesting that *arlRS* is upstream rather than downstream of *agr*. In contrast, *arlRS* induces expression of *sarA*. To the extent that *sarA* is a major repressor of protease production, this is consistent with the observation that protease activity is increased in an *arlRS* mutant[53]. Whether these effects are direct or indirect remains unclear.

Together, these results suggest that *arlRS* may be a key regulatory element that defines the "balance" between *agr* and *sarA*. Both of these regulatory elements have been implicated in biofilm formation, and *arlRS* has also been shown to have an impact in this regard. Specifically, inactivation of *arlRS* results in increased autolysis and an enhanced capacity to form a biofilm[52]. The fact that the biofilm phenotype appears to be independent of any effect on production of the *ica*-encoded poly-N-acetylglucosamine (PNAG)[54], together with the demonstration that extracellular DNA released from lysed *S. aureus* cells contributes to biofilm formation[55], suggest that increased autolysis may be responsible for the biofilm phenotype. However, the biofilm formed by an *arlRS* mutant is sensitive to exogenous proteinases, suggesting that this biofilm is also at least partially dependent on protein-protein interactions[54]. This is consistent with the observation that inactivation of *arlRS* results in dramatically increased amounts of extracellular and surface-associated protein A[53], both of which have been shown to contribute to *S. aureus* biofilm formation[56]. Finally, *arlRS* has been shown to promote the production of additional virulence factors including the exfoliative toxin and capsular polysaccharides, the latter being an indirect effect mediated through its positive regulation of MgrA production[57-59].

LytSR

Like *arlRS*, the *lytSR* two-component system is a negative regulator of *S. aureus* autolysis[60] and biofilm formation[61], and in fact *arlRS* is an activator of *lytSR* transcription[62]. These phenotypes are likely to be connected in that current models suggest that *lytSR*, together with CidR, collectively control the release of extracellular DNA (eDNA) by influencing expression of the *lrgAB* and *cidABC* operons, respectively, in modulating the production of murein hydrolases and consequently cell lysis[63]. Specifically, CidR activation of *cid* operon results in increased production of murein hydrolases, increased release of eDNA, and an increased capacity to form a biofilm, while activation of the *lrgAB* operon by LytSR has the opposite effects[55,64]. Although a cause-and-effect relationship between these phenotypes has not been proven, extracellular nuclease, whether applied exogenously or produced by *S. aureus*, has been shown to limit biofilm formation at least under certain *in vitro* conditions[29,64,65].

SrrAB

The *srrAB* two-component system was first identified based on homology with the ResDE two-component system in *B. subtilis*[66]. In response to oxygen stress, SrrAB represses expression of *agr* and the genes encoding certain exotoxins, including TSST-1[66]. However, it also represses transcription of *spa* and the production of protein A, which suggests that the impact of *srrAB* is not mediated directly through its regulation of *agr* but rather by direct interactions between the SrrA response regulator and the target genes themselves[67,68]. SrrAB also positively regulates expression of the *icaADBC* operon and production of PNAG, apparently by repressing transcription of the *ica*R-encoded repressor[67]. Whether the effect of SrrAB on the production of protein A or PNAG affects biofilm formation remains unknown, but the latter has been correlated with increased resistance to phagocytosis[47,69]. The link between oxygen availability, the activity of SrrAB, and the production of multiple types of virulence factors provides an important example of the link between central metabolic processes and virulence in *S. aureus*, a link that is also increasingly being made in the context of other *S. aureus* regulatory elements[70].

HssRS

The *hssRS* two-component system is an iron-responsive system that is highly conserved among Gram-positive pathogens including *B. anthracis*, *L. monocytogenes*, *S. epider-*

midis and *E. faecalis,* suggesting a conserved mechanism of iron acquisition among these organisms[71]. Iron is an essential nutrient for many bacterial species during infection[71,72]. However, free iron is severely limited in the human body but rather is complexed with a variety of iron-binding proteins. Therefore, in order for bacterial organisms to acquire iron they must have a mechanism for freeing complexed iron. *In vivo*, *S. aureus* can acquire iron in the form of heme, likely accessed *via* lysis of erythrocytes, using highly efficient transport systems that can move heme into the bacterial cytoplasm[73-75]. However, a high level of heme is toxic to the bacterial cell. To avoid toxicity, *S. aureus* senses heme by HssS resulting in HssR phosphorylation and binding to the promoter of *hrtAB*, which encodes an iron efflux pump that maintains intracellular heme homeostasis[76]. Whether there is cross-talk between this system and heme uptake systems, however, has not been demonstrated. In the absence of HrtAB, intracellular iron builds up causing a stress response characterized by the increased production of multiple virulence factors. Indeed, an *hrtAB* mutant is more virulent than the wild-type[71], likely due to the stress response induced by increasing intracellular heme.

Other two-component systems

The preceding discussion of two-component systems in *S. aureus* is by no means comprehensive, but it does summarize the impact of some of the best characterized systems. However, in the interest of inclusivity, we would note the existence of other, less well-characterized systems including KdpDE, which has been shown to link the AI-2/LuxS quorum-sensing system with capsule production[77]; VraSR, which induces a stress response to cell-wall inhibitors such as β-lactams and vancomycin[78]; GraSR, which aids in resistance to oxidative stress, heat stress, and vancomycin resistance[79]; BceAB, which is associated with altered susceptibility to bacitracin[7], and NsaRS, which plays a role in biofilm formation as well as cell envelope stability in response to cell wall and membrane disruption[80]. An additional two-component system that stands out from the others because it is the only one that is essential in *S. aureus* is WalKR (YycGF), which has been shown to be involved in peptidoglycan crosslinking and biofilm formation[81-83].

OTHER TRANSCRIPTIONAL REGULATORS

SarA-family

A primary class of transcriptional regulators that are not part of a two-component regulatory system, but do interact in multiple pathways with such systems, is the SarA family. The first gene encoding a member of this family, also identified in a screen of a transposon mutant library based on altered production of exotoxins, was designated the *sar*[84], which was subsequently changed to *sarA* based on identification of additional homologs now totaling 11[85]. Members of the SarA family have been shown to interact with each other forming part of a complex regulatory network controlling virulence factors (Figure 1). While all are winged-helix DNA-binding proteins, they can be divided into three structural families consisting of (1) relatively small, single-domain proteins (SarA, SarR, SarT, SarV, SarX and Rot); (2) larger, two-domain proteins (SarS, SarU, and SarY) in which each domain shares homology with the smaller homologs; and (3) small homologs with similarity to the MarR protein of Gram-negative bacteria (SarZ and MgrA)[85].

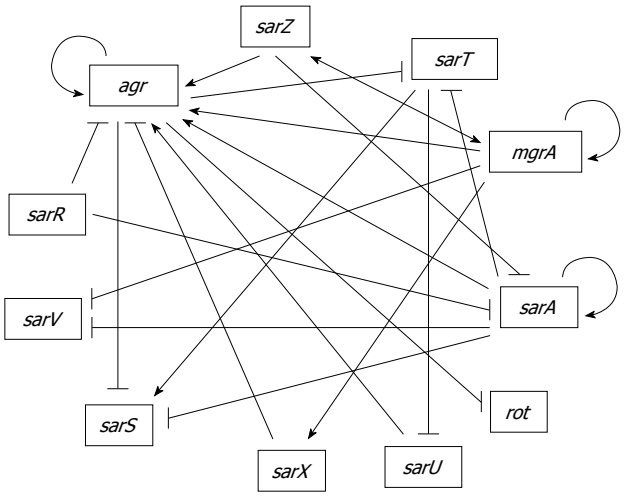

Figure 1 Proposed genetic regulatory network involving *sarA* family genes and *agr* in *Staphylococcus aureus*. The model is constructed from published studies[25,29,31,86,101,105,106,109,110,114,115,120-125,129,130,135,141,144] that are mostly based on a limited number of laboratory strains. Therefore, it may not be entirely applicable to all strains. Arrows indicate activation; blocked arrows indicate repression.

SarA

SarA, the prototype member of the SarA family, was identified in a screen of transposon-insertion mutants in the *S. aureus* strain DB based in part on its increased production of multiple exoproteins, a phenotype which clearly distinguished *sarA* from *agr*[84]. Based on this, it was proposed that *sarA* may function as a "counter-regulatory system to that of *agr*". This is consistent with the hypothesis that many regulatory functions in *S. aureus* are defined by the "balance" between *agr* and *sarA*, an issue that is discussed in more detail below in the specific context of biofilm formation. However, subsequent studies also confirmed that SarA binds to intergenic region between the *agr* P2 and P3 promoters[86,87] and is required for maximal transcription of *agr*[88]. Thus, it has become clear that SarA modulates the production of *S. aureus* virulence factors *via* both *agr*-dependent and *agr*-independent pathways.

The *sarA* locus is complex and includes three promoters (P1, P2, and P3) that drive the production of three transcripts (*sarB*, *sarC* and *sarA* respectively), with all three sharing the same termination site just downstream of the gene encoding SarA. The upstream *sarA* P2 and P3 promoters modulate the production of SarA by an unknown mechanism[89]. Specifically, inactivation of

P2 and P3 results in reduced production of SarA from the P1 promoter, and while the effect is relatively modest (approximately 2-fold), it appears to be functionally relevant with respect to both the *agr*-dependent and *agr*-independent pathways of SarA-mediated regulation[89]. This may account for the inability to demonstrate a difference between the three transcripts in complementation studies using a multi-copy vector targeting the *cna* promoter[90].

Both the signals that modulate SarA production and/or activity and the binding site for SarA are poorly defined. The DNA-binding activity of SarA is altered by redox state *in vitro*[91]. A recent report also demonstrated that SarA is phosphorylated by at least two serine/threonine kinases (Stk1/PknB and a poorly defined kinase encoded by SA0077) and that this also alters its DNA-binding capacity[92]. Two approaches have been taken to identifying the SarA binding site, with the first being alignment of the promoter regions of genes whose RNA products are altered in *sarA* mutants[93] and the second being the relatively unbiased approach of selective enhancement of systematic evolution of ligands by exponential enrichment (SELEX)[94], but generally speaking both approaches failed to define the characteristics of a definitive binding site beyond it being AT rich. It has been suggested on this basis that SarA may act as an architectural accessory protein rather than a classic transcription factor, a suggestion that is supported by the observation that SarA supports *E. coli* lambda phage integrase mediated site-specific recombination[91]. A recent report describing the interaction between SarA, SarR (see below) and *cis* elements within the *agr* promoter region also suggested that SarA binding may locally bend DNA in a fashion that brings AgrA into a favorable conformation to initiate transcription, particularly at the *agr* P2 promoter[87].

Irrespective of the mechanism involved, it is clear that inactivation of *sarA* results in major changes in the production and/or persistence of multiple RNA transcripts and that this has a global impact on the *S. aureus* virulon. Transcriptional profiling comparisons between the 8325-4 strain RN6390 and the clinical osteomyelitis isolate UAMS-1 demonstrate that this is somewhat strain-dependent, with the impact of mutating *sarA* in the latter being comparatively greater than the impact in the former[24]. RN6390 has defects in at least two genes that also have a major impact on global regulatory circuits. One of these is in *rsbU*, which results in reduced activity of the *sigB* regulon[47]. This is consistent with the observation that *sigB* has been shown to increase expression of *sarA* and reduce the level of RNAIII[95]. In this respect it is important to note that 8325-4 strains also have a mutation in *tcaR*, which results in reduced production of SarS[96], a SarA homolog also shown to influence the regulatory functions of SarA (see below).

As with *agr*, inactivation of *sarA* has been shown to attenuate virulence in multiple animal models of *S. aureus* infection including endopthalmitis, septic arthritis, osteomyelitis, and endocarditis[97-99]. Interestingly, all of these infections can arguably be said to be biofilm associated, an important observation given that inactivation of *sarA* has been consistently shown to result in a reduced capacity to form a biofilm[28]. In this respect it is also important to note that the impact of *sarA* on biofilm formation is opposite to that of *agr*[29], thus suggesting that the role of *sarA* in biofilm formation is independent of its regulation of *agr*. Indeed, in RN6390, a strain which expresses *agr* at high levels, inactivation of *agr* enhances biofilm formation in a manner that is reversed by concomitant inactivation of *sarA*, thus demonstrating that the impact of *sarA* is epistatic to *agr* in this context. Taken together, such results are consistent with the hypothesis that a primary determinant of the overall patterns of *S. aureus* virulence factor production is the "balance" between expression of *agr* and *sarA*[24], perhaps to the point of determining the relative capacity of different *S. aureus* strains to cause chronic, biofilm-associated infections *vs* acute, toxin-mediated disease.

There are several possible explanations for the biofilm-deficient phenotype of *sarA* mutants including the reduced expression of the *icaADBC* operon resulting in decreased production of PNAG, and the increased production of extracellular nucleases and proteases[29,31,64,65,100,101]. It seems unlikely that the decreased production of PNAG plays a predominant role given that inactivation of *sarA* has a greater impact on biofilm formation than inactivation of *icaADBC*[28]. Similarly, extracellular DNA has been shown to contribute to biofilm formation, but inactivation of the genes encoding *S. aureus* exonucleases has relatively little impact on the biofilm-deficient phenotype of *sarA* mutants[64,65]. In contrast, inactivation of the genes encoding extracellular proteases has a significant impact on the ability of *sarA* mutants to form a biofilm[24,29,102]. The effect of these proteases is presumably mediated *via* degradation of surface-associated proteins including FnbA, FnbB and protein A (Spa), all of which contribute to biofilm formation in *S. aureus* and have been shown to be produced in reduced amounts in *sarA* mutants owing to protease-mediated degradation[51,103,104].

Interestingly, the increased production of extracellular proteases has also been shown to result in the reduced accumulation of critical extracellular toxins in *sarA* mutants, at least under *in vitro* conditions[51]. These include α toxin and PSMs, both of which have been implicated as primary determinants of the hypervirulence of USA300 isolates[105]. Thus, while *agr* and *sarA* have opposite effects on the production of extracellular proteases, inactivation of either generally results in a toxin-deficient phenotype. This also suggests an alternative explanation for the reduced virulence of *sarA* mutants, although the relative contribution of these two *sarA*-dependent phenotypes in this respect remains to be determined.

Finally, the impact of SarA on exotoxin production has been shown to be heavily influenced by SaeRS, with the hyperactivity of SaeRS in Newman attenuating the increased production of extracellular proteases to a degree

that impacts the α toxin and PSM phenotypes of a Newman *sarA* mutant[51]. This, together with the impact of SarA on expression of *agr*, provides direct indications of the interactive role of SarA in *S. aureus* regulatory circuits. Additional interactions involving other SarA homologs are described below.

SarR

SarR was discovered based on its affinity for the *sarA*P2 promoter, with the binding of SarR repressing *sarA*[106]. In contrast, SarA binds its own promoters to enhance transcription, thus providing an example in which SarA and SarR serve competitive roles in modulating gene transcription. These two proteins also serve the opposing roles with respect to Agr since binding of SarA, together with AgrA, promote transcription from the *agr* P2 promoter whereas binding of SarR has the opposite effect[87]. It should be noted that SarR was originally shown to activate *agr* P2 promoter[107,108]. This discrepancy was attributed to the difference in *sigB*[87] but it is unclear how *sigB* reverses the effect. SarR promotes transcription of the proteases Aur and SspA, and may be involved in the SarA-dependent repression of these proteases, presumably also *via* competition for binding sites in the promoter regions[109]. In addition, SarR also binds to the *rot* promoter[107,108] but it is not known how SarR affects Rot. Thus, SarR plays an important but opposing role to SarA in both the *agr*-dependent and *agr*-independent pathways of SarA-mediated regulation. Based on these findings, SarR likely plays an important role in virulence due to its regulation of the well-characterized regulators SarA, Rot and Agr.

SarS

SarS (previously designated SarH1) was discovered using a search for proteins with affinity for the promoter region of *agr*-P3, *spa*, *hla* and *ssp*. However, SarS only affects expression of *spa* and *hla* but not RNAIII or *ssp*[110]. SarS is an activator of *spa* and a repressor of *hla* but it is repressed by Agr and SarA and activated by SigB and TcaR[96,111]. The fact that SarS is regulated by SigB and TcaR may explain why SarA affects *hla* transcription differently in RN6390, in which both SigB and TcaR are defective, than in other clinical isolates[111,112].

SarT/SarU

SarT was originally described by Schmidt *et al*[113] following a search for SarA homologs[113]. The *sarT* gene encodes a 118-residue protein and is present in certain strains of *S. aureus* including members of Clonal Complex 8 (CC8), to which CA-MRSA strains of the USA300 lineage and RN6390 belong, but absent in other clinically-relevant strains such as UAMS-1 (CC30 lineage)[113,114]. In the RN6390 background, *sarT* and *agr* are mutually repressive thus forming a negative feedback loop. In addition, *sarT* is also repressed by SarA[115]. Repression of *agr* by SarT was thought to explain the repression of *hla* by SarT in RN6390[115,116]. However, a later study shows that SarT represses *hla via sae* independently of *agr* and *rot* in strain COL[117]. A high level of *agr* in RN6390[118] may account for this difference. In RN6390, SarT also induces expression of protein A but indirectly through activation of *sarS*.

Adjacent to but divergently transcribed from *sarT* is *sarU*, whose expression is repressed by SarT[116]. Additionally, inactivation of *sarU* results in a reduction of both RNAII and RNAIII expression, suggesting a positive effect of SarU on *agr*. Because *sarT* has been shown to be repressed by *agr*, these relationships implicate a feedback loop involving SarT, SarU and RNAIII[116].

Recent studies have concluded that *sarT* and *sarU* are expressed at undetectable levels by northern blot[119]. However, deletion studies have revealed downstream effects of these genes, suggesting that they are expressed at very low but relevant levels[113,115,116]. For instance, it has been shown that a significant number of spontaneous non-hemolytic variants arise in biofilms that are phenotypically but not genotypically *agr* deficient. Transcriptional profiling of these variants found a 6-fold reduction in *sarU* suggesting SarU may be responsible for the *agr* deficiency[120]. These results imply that SarU may play a key role during biofilm-associated infections by modulating *agr*.

SarV

SarV was identified based on homology to SarA family. Both SarA and MgrA repress *sarV* gene expression. SarV is involved in regulation of autolysis, which may be part of the common pathway through which SarA and MgrA control autolysis[121]. Under laboratory conditions, *sarV* is poorly transcribed and the protein is not detectable in various strains in all phases of growth, likely due to repression by SarA and MgrA[119].

SarX

SarX was also identified based on sequence homology with the SarA family of transcription regulators. SarX has been shown to have maximal expression during the stationary phase of growth[122]. MgrA positively regulates *sarX* gene expression. SarX also acts as a repressor of the *agr* locus and can therefore regulate other genes *via* Agr[122]. SarX is highly expressed in RN6390 but is only expressed at very low levels in several tested strains[122] possibly due to difference in SigB in these strains. SarX has been shown to activate biofilm formation in *S. epidermidis*[123] raising the possibility that it may also have an effect on biofilm in *S. aureus*.

SarZ

SarZ was originally identified as a regulator of hemolysins and promotes virulence in both silkworm and mouse infection models[124]. SarZ positively regulates *agr* and *mgrA* expression but negatively regulates expression of *sarA*. SarZ affects surface proteins, toxins and biofilm through modulating the aforementioned global regulators as well as direct activation on SspA protease[125]. However,

SarZ binding to the promoters of various target genes is nonspecific[124,126]. In addition, SarZ activates SarS and is activated by MgrA[125]. SarZ and MgrA therefore interact in a positive feedback loop. SarZ expression is growth phase dependent, with maximum expression during early exponential phase[126]. Like MgrA, SarZ senses oxidative stress *via* a conserved cysteine residue providing another connection between metabolism and virulence[127,128].

MgrA

MgrA, also referred to as Rat and NorR, was identified in three independent laboratories[129-131]. MgrA regulates a multitude of virulence factors as well as antibiotic resistance[130,131]. Truong-Bolduc *et al*[130,132] initially described MgrA (NorR) as a regulator of NorA, which is a multidrug efflux pump providing quinolone resistance by direct DNA binding to the NorA promoter[130,132]. Binding of MgrA to the NorA promoter is dependent on phosphorylation by the kinase Stk1 (PknB), and RsbU is involved in dephosphorylation of MgrA leading to strain-dependent differences in MgrA function[133,134].

MgrA has been shown to up-regulate expression of 175 genes and down-regulate expression of 180 genes[59]. It was later shown that MgrA regulates *hla* and *spa* expression through *agr*-dependent and independent pathways[135]. In addition, MgrA has been found to repress biofilm formation, which is dependent on surface proteins, in part, through *agr*-dependent pathway and DNA release, probably by affecting LytSR and LrgAB[129,135,136].

MgrA has been shown to affect virulence in animal models of infection[59,137]. MgrA acts as a redox-switch as oxidation of the unique cysteine residue leads to its dissociation of MgrA from DNA[137]. Small molecules that disrupt the DNA-binding of MgrA had been shown to attenuate *S. aureus* virulence in mice[138] suggesting that MgrA could be a potential drug target.

Rot

Rot, repressor of toxins, is yet another SarA homolog[139]. It shares a high degree of sequence similarity to other members of the SarA family, but differs by its pI value (pI 5.0), which is more acidic than other SarA- homologs (pI values ranging from 8.5 to 10.7). Rot was first identified using transposon mutagenesis and screening for mutants capable of restoring the expression of α toxin and proteases in an *agr*-negative background[139]. Transcription of *rot* originates from at least three promoters and is growth-phase dependent[140,141]. Rot has an opposing effect on virulence gene expression by comparison to *agr*[139,142]. RNAIII blocks the translation of *rot* mRNA *via* an antisense mechanism, which explains why the regulatory function of Rot is only detected in *agr* deficient strains[20,143]. ClpX, a molecular chaperone, has also been shown to modulate Rot expression, likely by stimulating translation of the *rot* mRNA *via* a mechanism independent of RNAIII[144]. Rot has also been shown to repress α-toxin production by repressing the SaeRS two-component system[117]. In addition to toxins, Rot also positively regulates protein A[21,112,144,145].

AraC/XylS family transcription regulators

The *S. aureus* genome contains 6 ORFs with homology to the AraC/XylS family of transcriptional regulators. Of these, two have been characterized and demonstrated to play a role in biofilm formation[146,147]. Rbf was first identified using transposon mutagenesis in a screening for biofilm-deficient mutants and demonstrated to control biofilm in response to NaCl and glucose[147]. It was later determined that Rbf promotes biofilm formation *via* repression of *icaR*, a repressor of the *icaADBC* operon whose gene products synthesize PNAG. However, Rbf is unable to bind specifically at the *icaR* promoter[148,149] suggesting other regulators or post-translational modification of Rbf may be involved. Rbf has been shown to promote virulence in a murine foreign-body infection model[150].

Rsp, another AraC family regulator, has recently been characterized and shown to regulate biofilm formation[146]. However, Rsp differs from Rbf by repressing biofilm by negatively regulating surface proteins; in particular, FnbA, which has been shown to promote biofilm formation in the cell accumulation phase[151]. Interestingly, Rsp inhibits biofilm through repression of FnbA at the stage of primary attachment by direct binding to the promoter of *fnbA*[146].

CodY

CodY functions as a highly conserved regulatory transcription factor in low-GC Gram-positive bacteria and has recently been identified as a regulator of several virulence factors in *S. aureus*[152-154]. CodY acts in response to metabolite effectors such as GTP and the branched-chain amino acids isoleucine, leucine, and valine[153], all of which interact with a branched-chain amino acid domain on CodY, facilitating the direct binding of CodY to several promoters associated with virulence genes[155]. It is thought that CodY primarily acts as a negative regulator of virulence genes in *S. aureus*[156]. Microarray analysis and DNase footprinting of *codY* mutant clinical isolates have recently identified several negatively regulated targets of CodY including *agr*, *ica,* and *hla*[153,155]. The *agr* locus is responsible for regulation of many virulence factors and thus, the repression of this locus by CodY has profound phenotypic effects on the expression of virulence genes in *S. aureus*. For example, capsular polysaccharide production is repressed by CodY through *agr* as well as by direct promoter binding[155].

Apart from direct regulation of virulence genes, CodY also affects metabolic regulation in *S. aureus via* amino acid synthesis, carbon flow, nitrogen assimilation, and transport systems[153]. CodY is activated in nutrient-replete environments, repressing virulence factors and metabolic synthesis genes. For example, CodY is associated with repression of a lactate dehydrogenase (*ldh*1), which interconverts pyruvate and lactate[157]. *S. aureus* strains lacking *ldh*1 show significant attenuation of viru-

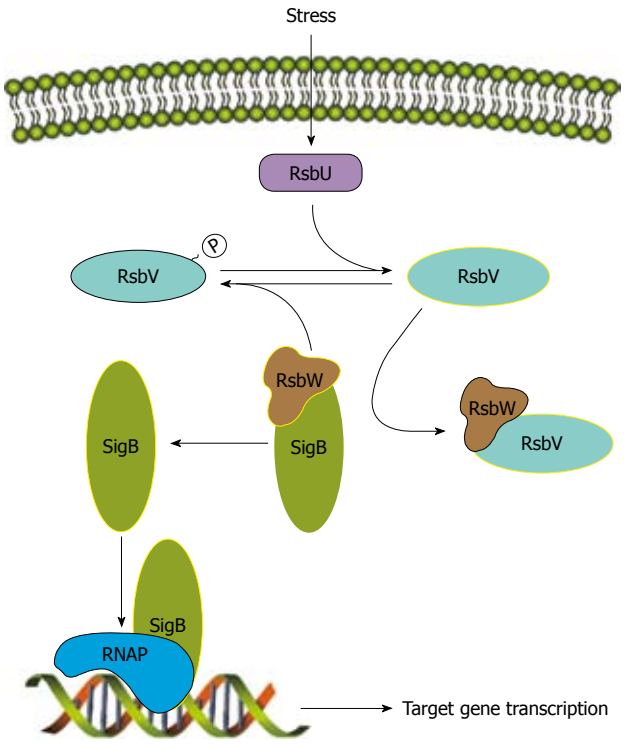

Figure 2 Post-transcriptional regulation of SigB. After stress-induction, RsbU de-phosphorylates RsbV, which can then bind specifically to RsbW thereby removing RsbW from SigB. Phosphorylated RsbV is inactive and therefore cannot bind RsbW. RsbW also promotes phosphorylation of RsbV to maintain its inactivity. RsbW binds to SigB to inhibit transcription by preventing SigB from complexing with the RNA polymerase (RNAP). Once SigB is free from inhibition by RsbW, it can complex with RNAP forming the holoenzyme and activate transcription of target genes. Active proteins are highlighted with yellow.

lence[157]. Thus, CodY is able to regulate virulence *via* direct binding of virulence gene promoters and *via* inhibition of metabolic regulatory pathways providing another regulatory link between metabolism and virulence[70,156].

CodY is repressed by the intracellular chaperone ClpC[40], possibly *via* ClpC-induced proteolytic degradation in association with ClpP. Although CodY acts as a repressor of virulence genes, it can also be negatively regulated under various environmental conditions, eliminating the repressive effect of CodY on virulence genes.

Sigma factors

Sigma factors are highly conserved among bacterial species. They provide promoter specificity to the RNA polymerase, and are highly regulated by anti-sigma factors *via* direct binding of the protein[158]. There are currently four identified Sigma factors in *S. aureus*: SigA, which is responsible for transcription of housekeeping genes; SigB, which is responsible for the transcription of stress-response genes; SigS, which controls expression of genes required for overall fitness and survival[2]; and SigH, which has a demonstrated involvement in competence and more recently, prophage integration and excision[159,160].

The most thoroughly studied of these is SigB, which is transcribed from the four-gene operon *rsbUVWsigB* that encodes an anti-sigma factor (RsbW), anti-anti-sigma factor (RsbV) and RsbU, an anti-RsbV phosphatase[95,161]. The regulation of SigB is very tightly controlled by RsbW, RsbV and RsbU (Figure 2). SigB controls expression of an array of genes responsible for the survival of hydrogen peroxide-induced stress and desiccation as well as production of the carotenoid staphyloxanthin and extracellular proteases[161-164]. SigB has also been demonstrated to aid in heat tolerance and resistance to cell-wall active antibiotics[165,166]. The repressive effect of SigB on V8 proteases positively regulates biofilm formation[162] because the presence of extracellular proteases has been correlated with the inability to form a biofilm[164]. SigB regulates its target genes either by recognizing a conserved sequence or by downstream regulators. For example, SigB effect on *sarA* or *agr* expression has been reported[95,167]. More recently, SigB has been shown to regulate several extracellular virulence factors and capsule through SpoVG[168,169], demonstrating a role for SigB in virulence as a response to stress.

CONCLUSION

In this review, we describe several regulators involved in virulence regulation. These represent only a fraction of all regulators encoded in the *S. aureus* genome. *S. aureus* is a pathogen that can cause a wide range of diseases and can infect almost every tissue. It is thus not surprising that a large number of regulators are needed to modulate the production of various virulence factors in different environmental conditions in the host. What is surprising is the high degree of complexity of the interactions among the regulators. Compounding the complexity is the finding that virulence genes in different strains often are regulated differently. The molecular mechanisms underlying some of the strain differences have been illustrated but most have not. Nonetheless, significant progress has been made toward understanding virulence gene regulation. However, most of the results have been obtained by *in vitro* studies. The big challenge that lies ahead would be to test the *in vitro* results in suitable animal models to better understand virulence gene regulation in pathogenesis. With the rise of antibiotic resistance and the prevalence of multi-drug resistant isolates, fully understanding the virulence regulation in pathogenesis may provide sound rationale for identifying regulators as potential targets for anti-staphylococcal drug therapies. Targeting a cellular factor not absolutely required for survival, such as a virulence regulator, may lessen selective pressures, and therefore resistance, while still attenuating virulence of the organism[170,171].

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