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Regulation of *Staphylococcus aureus* virulence

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Abstract

Staphylococcus aureus is a Gram-positive opportunistic pathogen that has evolved a complex regulatory network to control virulence. One of the main functions of this interconnected network is to sense various environmental cues and respond by altering the production of virulence factors necessary for survival in the host, including cell surface adhesins and extracellular enzymes and toxins. Of these *S. aureus* regulatory systems, one of the best studied is the accessory gene regulator (*agr*), which is a quorum-sensing system that senses the local concentration of a cyclic peptide signaling molecule. This system allows *S. aureus* to sense its own population density and translate this information into a specific gene expression pattern. Besides *agr*, this pathogen uses other two-component systems to sense specific cues, and coordinates responses with cytoplasmic regulators of the SarA protein family and alternative sigma factors. These divergent regulatory systems integrate the various environmental and host-derived signals into a network that assures optimal pathogen response to the changing conditions. This chapter gives an overview of the most important and best studied *S. aureus* regulatory systems and summarizes the function of these regulators during host interactions. The regulatory systems discussed include the *agr* quorum-sensing system, the SaeRS, SrrAB and ArlRS two-component systems, the cytoplasmic SarA-family regulators (SarA, Rot and MgrA), and finally the alternative sigma factors (SigB and SigH).

INTRODUCTION

Staphylococcus aureus is a Gram-positive commensal bacterium and opportunistic pathogen. The main sites of colonization are the skin and mucous membranes and approximately 30% of the healthy adult population are colonized by *S. aureus* (1). Although *S. aureus* is primarily a commensal microbe, it has the potential to cause a wide range of diseases that can vary considerably in severity. The most common problems are skin infections, and some of the most severe are bloodstream infections, endocarditis, osteomyelitis, and necrotizing fasciitis (2). In order to survive and adapt to different environmental niches, *S. aureus* has evolved an intricate regulatory network to control virulence factor production in both a temporal and host location manner (3). The regulatory machinery and virulence factors are known as accessory genes, since they are not essential for normal growth. These accessory factors are used to establish dominance in the host and contribute to the pathogenicity of *S.*

aureus, and they include cell surface components and proteins directly released into the extracellular environment. The functions of these molecules include adherence to host cells, evasion of host defenses, nutrient degradation and acquisition. These accessory genetic elements are encoded directly on the chromosome, and also on mobile elements that include phages, plasmids and pathogenicity islands.

S. aureus is a bacterial species with a conserved core genome (4), and its evolution is mainly driven through mutation and horizontal gene transfer. Mobile genetic elements, such as integrated bacteriophages (prophages), are one of the most common contributors to *S. aureus* strain-to-strain variation. Due to its low level of natural competence, bacteriophage transduction is a frequent mode of DNA transfer between *S. aureus* strains (5). Pantone-Valentine leucocidin (LukSF) and the immune evasion cluster (IEC) are two examples of virulence factors found on prophages (6, 7), with the latter being on a bacteriophage integrated into the *hly* gene (8). Other important bacteriophage encoded virulence factors include exfoliative toxin A (9, 10), cell wall-anchored virulence factor SasX (11), staphylococcal inhibitor of complement (*scn*) (12), staphylokinase (*sak*), chemotaxis inhibitory protein (*chp*) (13), and the enterotoxins encoded by *sea* (14), *selk2* and *selp* (15). Staphylococcal pathogenicity islands (SaPI) are another mobile genetic element that can encode pyrogenic toxins called superantigens. The superantigen genes encoded on SaPIs include toxic shock toxin (*tst*) (16), enterotoxin B (*seb*), and enterotoxin-like protein Q (*selq*) (15). Plasmids play an important role in the acquisition of antibiotic resistance of *S. aureus*, but there are also a few examples documenting their importance for the acquisition of virulence factors. To date it was shown that several toxins can be plasmid encoded, including exfoliative toxin B (17–19), enterotoxin D (20), the enterotoxin-like protein SER (21) and enterotoxin-like protein J (22). The focus of this chapter is the regulation of these virulence factors in *S. aureus*.

Regulation of *S. aureus* virulence factors

Within *S. aureus*, the regulation of the virulence factors is subject to a complex network that integrates host and environment derived cues into a coordinated response. Two-component systems (TCS) are one mechanism to perceive environmental changes and transform them into a regulatory program. For a typical TCS, an external signal activates the membrane-associated histidine kinase, leading to its autophosphorylation and subsequent phosphorylation of the response regulator. Once phosphorylated, the response regulator can bind to a specific DNA sequence motif, resulting in the alteration of target gene expression. Most strains of *S. aureus* encode sixteen different TCS (23, 24), one of which is essential (WalKR), and the other fifteen have been inactivated in multiple strains (25, 26). Some TCS such as *agrAC*, *saeRS* and *arlRS* are linked to *S. aureus* virulence and regulate a large number of host-impacting secreted proteins. The best studied of these regulatory systems is the accessory gene regulator (*agr*), which was first described in 1986 and encodes a quorum-sensing system that acts as a master virulence regulator (27). Coupled with these different TCS, *S. aureus* survives in the host environment using a suite of important cytoplasmic regulators (23). Among them are the SarA protein family of transcriptional regulators (SarA, Rot, MgrA, etc...), and the alternative sigma factors (SigB and SigH) being the most important. The goal of this chapter is to provide an overview of these regulatory systems

(summarized in Table 1), and the interplay and critical features of each system will be covered.

THE AGR QUORUM-SENSING SYSTEM

Architecture of the *agr* quorum-sensing system

The gene cluster that encodes the peptide quorum-sensing system in *S. aureus* is called the accessory gene regulator (*agr*) (Figure 1). The signal sensed by the *agr* system is an autoinducing peptide (AIP), which can be 7–9 amino acids in length and contains a 5-membered thiolactone ring between the C-terminal end and a conserved cysteine residue (3, 28, 29). The AIP signal accumulates in the extracellular environment, and once it reaches a critical concentration, usually at a “quorum” cells in the population, the system is activated. *S. aureus* employs the *agr* system to adapt to changing environmental conditions during growth and to regulate virulence (27, 29, 30). The *agr* system consists of two adjacent transcripts, called RNAII and RNAIII, whose expression are driven by the P2 and P3 promoter respectively (31). The RNAII transcript is an operon of four genes, *agrBDCA*, that encode the machinery of the quorum-sensing system, and the RNAIII transcript is the major effector and regulates the expression of most *agr*-dependent target genes. As shown in Figure 1, AgrD is the ribosomal peptide precursor of AIP (32) and is proteolytically processed by AgrB, an integral membrane-bound peptidase (33, 34). AgrB-mediated cleavage of AgrD results in the formation of an enzyme-bound thiolactone intermediate (33–35), and through an unclear mechanism this structure is transported across the membrane. The type I signal peptidase SspB performs the final processing step to release the mature AIP into the extracellular environment (36). Once outside the cell, AIP is then sensed by AgrC, the membrane-bound histidine sensor kinase of the AgrCA TCS. The binding of AIP to the AgrC receptor leads to histidine autophosphorylation (37), and this signal is relayed to the aspartate receiver on the response regulator AgrA (38). The phosphorylated AgrA can then bind to the P2 and P3 promoters to drive expression of RNAII and RNAIII, respectively (39). Expression of RNAII, which encodes for all components of the *agr* system effectively leads to a positive feedback loop. This autocatalytic regulation is a hallmark of quorum-sensing systems and enables *S. aureus* to readily produce exoproteins, even though growth is slowed down (38).

AIP types

To date, four different classes of AIP structures are known to exist in *S. aureus*. Their length ranges from 7 amino acids (type III), and 8 amino acids (type I and IV), to 9 amino acids (type II) (Figure 2). The AIP type I and type IV are the most conserved, only differing in one amino acid, and as a result, they function interchangeably (40). Early studies on *agr* revealed that the AIPs can cross-inhibit the function of an AgrC receptor from another *S. aureus* strain, in a mechanism termed “*agr* interference” (41). This cross-talk gave rise to three functional AIP groups, each of which can effectively cross-inhibit other *agr* systems within *S. aureus* (41), resulting in an intriguing mechanism of intraspecies signaling (Figure 2). The divergence among the four AIP types stems from a highly variable region that spans the C-terminus of *agrB*, the whole *agrD* gene, and the N-terminal region of *agrC* (42). These differences also explains the variability of the AgrC protein and its specificity for its cognate

AIP (41, 42). As mentioned above, the hypervariable region responsible for the four AIP types also reaches into the coding sequence of *agrB*, which suggest that AgrD processing might also be type specific. Indeed, it was demonstrated that AgrB of the AIP type II system can only process its cognate AgrD, while AgrB of the AIP type I and type III system are not able to process the type II AgrD (41, 43).

Agr regulon

AgrA directly regulates the expression of RNAII and RNAIII from the P2 and P3 promoters, respectively (Figure 1). This was long thought to be the only relevant regulatory role of AgrA, until a study compared gene regulation between wildtype, an *agrA* mutant, and a RNAIII mutant (44). It was discovered that AgrA also regulated the phenol-soluble modulins (PSMs) genes, which have received considerable attention in recent years for their involvement in *S. aureus* virulence (45–47). Electromobility shift assays demonstrated that AgrA indeed is capable of directly binding the promoters of the alpha- and beta-PSM encoding operons (44). The main effector of the *agr* quorum sensing system is RNAIII, a regulatory RNA that also encodes the delta-hemolysin gene (*hld*) (48), also known as delta-toxin. Interestingly, the translation of *hld* is delayed by one hour after RNAIII transcription by an unknown mechanism (49). Among the genes upregulated by RNAIII are many well characterized virulence factors, including alpha-toxin (Hla), serine proteases (SplA-F, SspA), cysteine proteases (ScpA, SspB), gamma-hemolysin (Hlg), leucocidins (LukAB, lukGH), and lipase (Geh) (30, 50, 51). Alpha-toxin is one of the most prominent regulated toxins and is an important virulence determinant in skin and soft tissue infections (52, 53), pneumonia (54) and endovascular infections (55). In contrast, some surface proteins including protein A (Spa), cell wall secretory protein (IsaA), and surface receptors (MnhA, MnhF and MnhG) are downregulated by RNAIII (30, 50, 51). Among these surface proteins, protein A stands out due to its dominant role in pathogenicity in several different types of *S. aureus* infections, including pneumonia (54, 56), bloodstream infections (57), and septic arthritis (58).

RNAIII enacts its regulatory role either directly on the transcriptional level by modulating transcription initiation, or at the post-translational level by interacting with the target gene transcript (48). RNAIII is a large regulatory RNA that has a complex secondary structure with several C-rich hairpins, which are important for the interaction of RNAIII and its target mRNAs (59). One well studied example is the positive regulation of alpha-toxin (*hla*) regulation by RNAIII. The *hla* mRNA normally forms a hairpin loop that prevents the ribosome from accessing the ribosome-binding site. RNAIII can bind to the *hla* mRNA, relieving the hairpin loop structure and allowing the ribosome to recognize the binding site for translation initiation (51). In some occasions, post-transcriptional control by RNAIII results in translational inhibition, rather than promotion. As one example, RNAIII exerts its regulatory activity on *spa* (Protein A) gene expression at the post-transcriptional level by two distinct mechanisms. The first mechanism is by direct RNA-RNA interaction with the *spa* mRNA, which inhibits access to the ribosome binding site, as well as the *spa* start codon, preventing the initiation of translation (60). The complex formed between RNAIII and *spa* mRNA is also a substrate for RNaseIII. Thus, RNAIII can also inhibit Protein A production by enhancing the degradation of *spa* mRNA (60). Although RNAIII is considered the major

effector of the *agr* system, regulation of many genes is achieved indirectly through the global regulator Rot (61). Rot is part of the SarA family of cytoplasmic regulators, and Rot's regulatory mechanism is detailed later in this chapter. Similar to the Protein A mechanism, RNAIII controls Rot expression by blocking the ribosome-binding site (59, 62, 63), facilitating *rot* mRNA cleavage by RNaseIII directly at this site (59, 63). Taken together, when the *agr* system activates, RNAIII levels are high, which in turn prevents the translation of Rot and alters the expression of downstream target genes (64).

Impact of environmental and host stimuli on *agr* function

As the *agr* system plays a central role in pathogenesis, *S. aureus* has evolved many different strategies to fine tune the expression of *agr*, allowing it to adapt quickly to changing conditions. These *agr* modulating factors include environmental cues, other *S. aureus* regulatory proteins, as well as host-derived stimuli. The following sections outline some of these various factors and the mechanisms through which they influence the *agr* quorum-sensing system.

Environmental cues.

One of the first abiotic factors that have been described to influence the *agr* system is pH. It was shown that acidic pH inhibits *agr*, which can either be achieved through the catabolism of glucose (65), or by directly lowering the pH of the growth medium in the absence of glucose (66). Interestingly, alkaline pH also represses RNAIII transcription, and taken together, it is evident that maximum *agr* activity is observed near neutral pH (67). Reactive oxygen species (ROS) can also influence the *agr* activity. Neutrophils are some of the first responders to *S. aureus* infection, and bombard the bacteria with oxidative killing mechanisms. Myeloperoxidase transforms H₂O₂ and chloride to HOCl (bleach), which modulates the *agr* system by oxidizing the methionine side chain of AIP-I and -IV (Figure 2), rendering the AIPs ineffective (68). Oxidative stress caused by ROS can also induce disulfide bond formation in AgrA, leaving it incapable of binding its target promoters (69). In addition, indirect regulation of *agr* activity by ROS is achieved by altering the activity of the AirSR (70) and the SrrAB TCS (71), which is discussed further in this chapter.

Host derived factors.

Host serum was one of the first identified inhibitors of the *S. aureus agr* system (72). In follow-up studies, it was found that apolipoprotein B (ApoB) within serum is primarily responsible for this phenotype (73, 74). ApoB sequesters AIP signal and prevents *agr* activation. In the host, *S. aureus* obtains iron from hemoglobin (75), but hemoglobin in turn can impact *agr* function. Initially, this was observed using the hemoglobin α and β chains, which inhibited exotoxin production and increased Protein A levels, suggesting that the *agr* system was repressed (76). Later studies demonstrated that indeed hemoglobin downregulates the *agr* P3 promoter activity, and that this can happen during colonization or infection (77).

Regulatory proteins.

There are many studies defining how *S. aureus* regulators modulate *agr* activity, and a comprehensive list of these regulators impacting *agr* can be found in Table 1. Of these regulators, CodY, MgrA, and SarA are covered in more detail here. CodY is a transcription factor present in several low G+C Gram-positive bacteria and senses the intracellular concentration of branched-chain amino acids and GTP. It regulates the cells response to nutrient limitation and metabolic stress (78). In a *codY* mutant, expression of the *agr* locus is upregulated (79). The *agr* locus is overexpressed in the exponential growth phase in a *codY* mutant, which correlates with accumulation of AIP and the dependency on phosphorylated AgrC and AgrA. CodY only weakly binds to the P2 and P3 promoter regions, and instead binds to a region within *agrC* that contains the P1 promoter (79, 80). Although the P1 promoter is weak and only drives *agrA* expression, the binding of CodY to this region could explain the negative regulation of *agr* function. In contrast to CodY, MgrA is a positive regulator of *agr* expression and belongs to the SarA-family of regulators. Two independent studies have shown that an *mgrA* mutation results in a decrease of RNAII and RNAIII transcripts (81), or even complete loss of RNAIII production and P3 promoter activity (82). However, a subsequent microarray study did not observe an impact of MgrA on RNAIII production (83), and a more recent RNAseq study had the same observation (84). These studies used different strains and therefore the regulation of *agr* by MgrA seems to be highly strain specific. Finally, SarA is a positive regulator of *agr* activity and one of the best studied virulence regulators in *S. aureus*. SarA exerts its positive regulation over *agr* by binding to the P2 and P3 promoter regions and subsequently activating transcription of RNAII and RNAIII (85, 86). A comprehensive description of SarA's role in *S. aureus* virulence regulation is given in a later section of this chapter.

Importance of *agr* during infection

Numerous studies have shown that the *agr* system is required for different animal models of infection. Strains with *agr* mutations show smaller lesion sizes and reduced bacterial load in murine skin infection models compared to wildtype strains (28, 87–90). These *agr* mutants are also attenuated in acute murine pneumonia models (54, 56, 89, 91), and they were shown to have decreased rates of infective endocarditis in rabbit models (92). Additionally, *agr* mutants display a decreased ability to establish osteomyelitis in a rabbit model (93). Using a P3-*lux* bioluminescence reporter strain, the dynamics of *agr* activation have been monitored in real-time during infection (90, 94, 95), and there is evidence that *agr* activation occurs in repeating waves *in vivo* (94).

TWO COMPONENT SYSTEMS

The SaeRS TCS

First described in 1994, the *sae* (*S. aureus* exoprotein expression) locus was initially characterized as a regulator of exoprotein production (96, 97). The SaeRS TCS consists of the histidine kinase SaeS and the SaeR response regulator (96–98). The operon encoding the SaeRS system contains four genes, *saeP*, *saeQ*, *saeR* and *saeS* (Figure 3). Expression of *saeR* and *saeS* is driven by the constitutive P3 promoter, which is located within the coding region of *saeQ* (99, 100). The P1 promoter is autoinduced by SaeRS and drives the

expression of all four genes (101). Although expression of the SaeRS system from the P1 promoter is much stronger than expression driven by the P3 promoter, the overall SaeRS activity is not elevated. This is explained by the fact that SaePQ forms a complex with SaeS, leading to enhanced phosphatase activity (102), and resulting in reduced signal transduction between SaeS and SaeR. Topological studies of the SaeS sensor kinase revealed a nine-amino acid extracellular loop that plays an important role in signal transduction. Three amino acids in the extracellular loop (M31, W32 and F33) were found to be important for SaeS activity. Interestingly, site-directed mutation in M31 and F33 abolished the ability of SaeS to respond to α -defensins (HNP-1) (103), which activates the SaeRS system at sub-inhibitory concentrations (104). Interestingly, a mutation of SaeS W32 still allowed a response to HNP-1. Exposure to human neutrophils showed that changing the aromatic amino acids (W32 and F33) in the anchor region of SaeRS did not inhibit its activity, while mutating M31 rendered SaeS non-responsive to neutrophils (103). In general, *S. aureus* wildtype survives better after neutrophil uptake compared with a *saeRS* mutant strain (105), indicating a crucial role for SaeRS in the sensing and defense against neutrophils.

The SaeRS two-component system drives the expression of several important exoproteins (Figure 3), including coagulase, alpha-toxin, β - and γ -hemolysins, staphylococcal immunoglobulin binding protein, Pantone-Valentine leucocidin (LukGH), TSST-1, exfoliative toxin and nuclease (97, 98, 105–107). The latter two were also shown to be SaeRS regulated *in vivo* during infection (107, 108). Across *S. aureus* strains, the sensor kinase SaeS has polymorphisms and the three known variants are designated as SaeS^P, SaeS^{SK} and SaeS^{SKT}. The SaeS^P variant, which is present in the *S. aureus* strain Newman, leads to SaeRS hyperactivity and higher transcription rates of numerous output genes, including *fnbA*, *coa*, *sib*, *efb*, *fib*, *eap* and *sae*, while other target genes like *hla*, *hly* and *cap* are not sensitive to the SaeS^P variant (109). Besides its autoinducing regulation, the SaeRS system is regulated by environmental cues and host specific signals. Low pH (5.5) as well as high salt concentration (1M NaCl) inhibit *sae* P1 promoter activity (66, 104). The inhibitory effect is relieved in a *saeP* mutant (110), suggesting that SaeP lipoprotein contributes to this regulatory mechanism. Metals, specifically copper and zinc, interfere with the signal transduction from SaeS to SaeR by inhibiting the SaeS kinase activity. Interestingly, zinc-bound calprotectin, a neutrophil intracellular protein protects the Sae system from metal mediated inhibition through an indirect mechanism (111). In addition, the human skin derived fatty acid *cis*-6-hexadecanoic acid was reported to inhibit the SaeRS system by an unknown mechanism (112).

The SrrAB TCS

The SrrAB (staphylococcal respiratory regulator) two-component system, also known as SrrSR, was first described as a global regulator of virulence factor production under low oxygen conditions (71, 113). The *srrAB/srrSR* locus encodes a histidine kinase SrrB (SrrR) and the paired response regulator SrrA (SrrS). The SrrAB TCS was shown to be important for resistance to nitrosative stress (114). More recent studies have shown that SrrAB also confers H₂O₂ resistance during high dioxygen-dependent respiration, which is achieved by the positive regulation of the H₂O₂ resistance genes *kat*, *ahpC* and *dps* (115). *S. aureus* *srrAB* mutants are also attenuated in their fermentative biofilm forming capacity. This

phenotype is in part due to the decreased abundance of autolysin (*atlA*) transcript in a *srrAB* mutant strain, resulting in impaired autolysis and eDNA accumulation under fermentative conditions (116).

In regards to other global regulatory effects, the *agr* system, protein A (*spa*), and TSST-1 are repressed by SrrAB, especially under low-oxygen conditions (71). The observed repression is a result of a direct interaction of SrrA with the respective promoters (117). Besides the aforementioned virulence factors, the SrrAB TCS is also a major driver of *ica* operon expression under low oxygen conditions, and this operon encodes the polysaccharide intercellular adhesin (PIA). An *srrAB* mutant is impaired in a neutrophil killing assay, suggesting that SrrAB-dependent PIA production may protect *S. aureus* against non-oxidative killing by neutrophils (118). Transposon sequencing (TnSeq) analysis revealed SrrAB to be essential for *S. aureus* survival in an osteomyelitis model, which could be rescued by neutrophil depletion (119). SrrAB was also found to be a positive regulator of the phosphatidylinositol-specific phospholipase C (PI-PLC) enzyme. PI-PLC is important for *S. aureus* survival in human blood and in human neutrophils (25), which could in part explain the importance of SrrAB for defense in these environments.

The ArlRS TCS

Bacteria can undergo autolysis to remodel their cell walls by the action of murein hydrolases, which hydrolyze various components of peptidoglycan (120). The ArlRS (autolysis regulated locus) TCS was first described as a negative regulator of the autolytic process (121, 122) and acts as a repressor of *agr* by downregulating RNAII and RNAIII expression (121). More recently it was observed that ArlRS is a repressor of autolysis in methicillin-sensitive *S. aureus* (MSSA) strains (122, 123), but not in methicillin-resistant strains (MRSA), including hospital- and community-acquired MRSA strains (123). In terms of virulence factors, ArlRS was reported to be a positive regulator of extracellular proteolytic activity, specifically serine protease activity (122). Serine proteases are involved in *S. aureus* virulence due to their ability to cleave antibodies and block their activity. This was for example demonstrated for the V8 serine protease of *S. aureus*, which cleaves immunoglobulin G in vitro (124). Although early reports demonstrated differential toxin regulation between wildtype and *arlRS* mutant strains, this was not observed in other studies using clinical isolates (125), supporting the notion that some of this reported regulation is highly strain specific.

In addition to *S. aureus* virulence, a microarray study showed that ArlRS represses sugar uptake systems and activates genes involved in amino acid utilization as carbon source (126). These early results were corroborated by a recent study, which showed positive regulation of genes involved in amino acid catabolism by ArlRS in the presence of calprotectin, an antimicrobial protein that starves bacteria for manganese and zinc (127, 128). A functional ArlRS system was necessary to facilitate resistance against calprotectin mediated manganese starvation. Interestingly, manganese sequestration by calprotectin inhibited growth of *S. aureus* when glucose was utilized as the main energy source, but not when amino acids served as the primary energy source. This implies that glucose utilization results in higher demand of manganese compared to amino acid utilization. Therefore *S.*

aureus protects itself from calprotectin mediated manganese starvation by switching its carbon source and this process seems to be regulated by the ArlRS TCS (122).

Recently it was demonstrated that the ArlRS TCS is required for *S. aureus* clumping in the presence of fibrinogen (125). *S. aureus* uses surface proteins, such as ClfA and ClfB, to interact with the abundant, dimeric fibrinogen molecule, bringing cells together as a “clump” in order to survive in the host (129). The inactivation of *arlRS* results in the dramatic upregulation of the large surface protein Ebh, which is thought to inhibit clump formation. Indeed, the *arlRS* mutant strain also showed lower virulence in a rabbit model of sepsis and endocarditis, and this phenotype was partially restored by mutation of *ebh* (125). A subsequent study revealed that ArlRS directly regulates MgrA (84), which is the primary controller of the clumping mechanism. Through RNAseq analysis, a total of eight large surface proteins (Ebh, SraP, Spa, FnbB, SasG, SasC, FmtB, and SdrD) were found to be repressed by MgrA (more on this regulator below). Depending on the strain, a deletion of SraP, SasG or Ebh (in combination) within an *mgrA* mutant background restores the clumping phenotype to wildtype levels (84). The fibrinogen mediated clumping phenotype of *S. aureus* was shown to have a major influence on *agr* signaling. Clumping creates a microenvironment in which the AIP concentration is increased and thus quorum-sensing is uncoupled from the actual bacterial density. This results in a boost in *agr* activation and subsequent production of virulence factors, leading to increased bacterial burden as well as host morbidity and mortality (130).

THE SARA PROTEIN FAMILY

SarA

SarA is the prototypical member of the SarA protein family in *S. aureus*. As outlined in Figure 4, SarA is expressed from three different promoters, leading to expression of three overlapping transcripts (131). SarA binds to its own promoter and auto-up-regulates its own expression (132). It is negatively regulated by the SarA-like protein SarR (133), and also negatively regulated by the alternative sigma factor σ^B (133, 134). One of the ways SarA exerts its global regulatory function is via the upregulation of the *agr* (Figure 4), resulting in enhanced expression of toxins and the repression of Protein A (*spa*) (135). More specifically, SarA significantly boosts the expression of the *agr* system from the P2 promoter (136). Besides indirect regulation through the *agr* system, SarA also exerts post-transcriptional regulation of targets (e.g. *spa*) by binding and altering the mRNA turnover (137).

Several studies have shown that SarA is a strong repressor of protease production, which effects several phenotypes, such as biofilm development (138), and the accumulation of proteins important for *S. aureus* virulence (139). An *in vivo* study showed that a *sarA* mutant of the USA300 strain LAC was attenuated in a murine infection model. The *sarA* mutant displayed increased extracellular protease production, which facilitate the degradation of extracellular virulence factors. Subsequently this phenotype could be reversed by eliminating extracellular protease activity (139). Another interesting aspect of SarA-mediated regulation is the decreased accumulation of alpha-toxin and PSMs in a USA300 strain. The altered virulence factor levels could be correlated with the increased extracellular degradative protease activity of the strain, rather than a direct regulatory effect of SarA on

either *agr* or *hla*. In contrast, mutation of *sarA* in strain Newman did not impact PSM or alpha-toxin accumulation (140), which might be explained by the fact that strain Newman has the SaeS^P variant, leading to constitutive activity of the SaeRS system. A knock-out mutation of *sarA* also leads to significant reduction of cytotoxicity against osteoblasts and osteoclasts, and reduced osteomyelitis in a murine model. This phenotype is thought to be mediated by the SarA-regulated production of α -PSMs (141).

Besides these regulatory functions, SarA also directly up-regulates other virulence factors, including toxic shock syndrome toxin 1 (TSST-1) through direct promoter binding (142, 143). Interestingly, the same research group identified SarA as a negative regulator of TSST-1 expression in another *S. aureus* strain background (134), suggesting TSST-1 regulation by SarA to be strain specific. Exfoliative toxin A and B, the causative agents of staphylococcal scalded-skin syndrome, are both downregulated by SarA *in vitro*. Negative regulation of ETB, but not ETA, by SarA was also observed in a neonatal mouse model (107). Another facet of SarA mediated regulation is the recently discovered repression of the regulatory small RNAs (sRNAs) *srn_3610_sprC*, known to attenuate *S. aureus* virulence in an animal infection model (144), and *srn_9340* (145). Repression is achieved by direct binding of SarA to the respective promoters of the two sRNAs, which consequently blocks RNA polymerase loading (145).

SarR

SarR is 13.6 kDa homolog of the SarA regulator protein and is a negative regulator of *sarA* expression (133) (Figure 4). SarR accomplishes this regulation by binding to all three promoters of the *sarA* locus (146). SarR also acts as a negative regulator of *agr* expression from the P2 promoter, and SarR binds better to the *agr* promoter than SarA. The result is that SarR can displace SarA and maintain negative regulation over *agr* function (136).

SarT and SarU

Two other members of the SarA protein family are encoded by the adjacent and divergently transcribed genes *sarT* and *sarU* (147, 148). Transcription of the *sarT* gene is regulated by *agr* and *sarA*, both of which repress *sarT* expression. While SarT has only minimal effect on SarA and SarR expression, the mRNA levels of the *agr* effector RNAIII are increased in a *sarT* mutant (147). Therefore, SarT and the *agr* system seem to interact in a negative feedback loop. Besides this regulation, SarT also represses *hla* (147). In addition, SarT is also a negative regulator of the *sarU* gene through binding promoter region. In contrast to a *sarT* mutation, the activities of the RNAII and RNAIII promoters are decreased in a *sarU* mutant when measured with a GFP reporter plasmid (148). In terms of the *agr* system, SarT and SarU play opposite roles in the regulatory mechanism. SarT production is also regulated by a small-noncoding RNA called ArtR. The ArtR transcript binds to the 5' untranslated region of the *sarT* mRNA, promoting its degradation (149). Interestingly, ArtR itself is positively regulated by *agr* and thus might be the mediator of *sarT* repression by *agr*.

SarH1

SarH1, also known as SarS (150), is a 29 kDa protein from the SarA family. Like other SarA family proteins, its regulation circuit is interwoven with other SarA family proteins and the

agr system. Expression of SarH1 is strongly repressed by SarA as well as *agr* (150, 151), while it is upregulated by SarT (152). On the post-transcriptional level, SarH1 is regulated by GdpS. The mRNA product of the *gdpS* gene directly interacts with the 5' UTR of *sarH1*, resulting in a more stable *sarH1* mRNA (153). SarH1 itself acts as a repressor of *hla*, and it also acts as a positive regulator of *spa*, which is located directly downstream the *sarH1* locus (151). The regulation of *spa* seems to be organized in a cascade. The *agr* system regulates SarT, which upregulates SarH1 and in turn positively regulates *spa* production (152). Another virulence factor regulated by SarH1 is exfoliative toxin. Two different exfoliative toxins, ETA (*eta*) and ETB (*etb*), are expressed by *S. aureus* strains. Of these, SarH1 downregulates production of ETA, but it does not affect ETB production. ETB and ETA production are also negatively regulated by SigB and SarA, while *saeRS*, *arlRS* and the *agr* system act as positive regulators (107). A recent study showed that SarH1 might also be involved in the spread of antibiotic resistance via SCCmec. SarH1 can upregulate the chromosome cassette recombinases A and B (CcrAB), which are responsible for the excision of SCCmec (154), which could possibly lead to its horizontal transfer.

Rot

The “repressor of toxins” (Rot) regulatory protein is a 15.6 kDa member of the SarA-like family. The name is derived from the fact that mutation of the *rot* locus in an *agr*-null background restores toxin production and protease activity, and consequently restored virulence in a rabbit model of endocarditis (61, 155). In terms of toxin regulation, Rot acts as a repressor of enterotoxin B (*seb*), alpha-toxin (*hla*), proteases encoded by the *spl* and *ssp* operons, as well as lipase (*geh*). Enterotoxin B is directly repressed by Rot through binding to the *seb* promoter (156). The repression of *hla* on the other hand is exerted indirectly via the SaeRS TCS. Rot represses *sae* transcription from the P3 promoter, which then leads to reduced *hla* expression (157).

Rot also acts as a positive regulator of multiple virulence factors. Protein A (*spa*) and the SarA-family protein SarH1 (158) are upregulated by Rot, and by directly binding to its promoter, Rot can positively regulate superantigen-like proteins (Ssl) (159). A subsequent study demonstrated that Ssl production is also dependent on the SaeRS TCS, which synergizes with Rot to activate the Ssl promoter (160). The Rot regulon overlaps with the *agr* system, and indeed Rot acts as an intermediate regulator of *agr* function. As described earlier in this chapter, RNAIII, the main effector of the *agr* system, blocks the translation of the *rot* gene (59, 62). Besides RNAIII, Rot is also repressed by SarA, through direct binding to the Rot promoter (161), and by the sigma factor B (σ^B) during stationary growth (63). On the post-transcriptional level, Rot production is also dependent on the presence of the ClpX chaperone (162).

MgrA

MgrA is an important member of the MarR (multiple antibiotic resistance regulator)/SarA protein family. MgrA is a positive regulator of 175 genes and a negative regulator of 180 genes in *S. aureus* strain Newman, as shown in a microarray study (83). Among these regulated genes are several encoding for virulence factors, including alpha-toxin, coagulase, Protein A, nuclease, extracellular serine proteases, and capsule production (163, 164). The

regulation of genes responsible for capsule production (*cap5*) has also been implicated in infective endocarditis, while alpha-toxin production was not MgrA-dependent in the same model (163). MgrA also regulates the expression of SarZ, which itself controls exoprotein production (165). Looking at the regulatory pattern of MgrA, it is evident that exoproteins are positively regulated and surface proteins are negatively regulated by MgrA (83), with some parallels to the *agr* system. Related to this point, the *mgrA* gene is transcribed from two different promoters (P1 and P2), and the mRNA transcript derived from the P2 promoter is stabilized by RNAIII (166).

In addition to RNAIII, MgrA production is regulated by another small RNA-mediated mechanism. The non-coding RNA RsaA represses MgrA production by interacting with its ribosome-binding site, making a loop-loop interaction with the *mgrA* mRNA (167, 168). The activity of MgrA is also regulated on the post-translational level via the phosphorylation of a conserved cysteine residue. This phosphorylation is mediated by the Stk1-Stp1 proteins, a eukaryotic-like kinase-phosphatase pair. Deletion of Stp1 results in elevated protein phosphorylation on cysteine residues and significantly reduces virulence in mouse infection models (169). The phosphorylation status of MgrA also plays a key role in the regulation of the NorA and NorB efflux pumps, which confer resistance to several antibiotics. Phosphorylated MgrA is able to bind to the NorB promoter, acting as a repressor. and unphosphorylated MgrA binds to the NorA promoter, acting as an activator (170–173). Further studies on MgrA suggest it has the ability to regulate the *lytRS*, *lrgAB* and *arlRS* TCS, which are all involved in *S. aureus* autolysis (81). MgrA was called Rat (regulator of autolysis – in answer to “Rot”) in early studies due to its role in autolysis regulation.

Several studies have shown that MgrA plays an important, but conflicting role in *S. aureus* biofilm formation (Figure 5). A mutation of *mgrA* has been reported to both promote (84, 174, 175) and repress biofilm formation (176). The disparity is most likely explained by the use of different *S. aureus* strains in these studies. The downregulation of surface proteins by MgrA has been corroborated in a recent study, which established a link between MgrA and the clumping activity of *S. aureus* in presence of fibrinogen (84). As described above (in the ArlRS section), MgrA represses eight large surface proteins, and of these Ebh, SraP and SasG are important in preventing clumping with fibrinogen. Fibrinogen mediated clumping facilitates increased *agr*-mediated virulence factor production and de-couples quorum-sensing from the actual cell density (130). This can potentially be detrimental for *S. aureus*, as virulence factor production is often a trigger for host defense. Thus MgrA might act as a fail save switch for pre-mature virulence factor production. Relating to biofilm development, the de-repression of SasG in an *mgrA* mutant strain promotes biofilm formation (84).

ALTERNATIVE SIGMA FACTORS

SigB

Sigma factors confer promoter specificity to the RNA polymerase holoenzyme and are required for transcription initiation. While the primary sigma factor controls housekeeping functions (177), alternative sigma factors regulate the response to changing conditions and allow *S. aureus* to adapt to different environments. Sigma factor B (σ^B) is known to respond to different stresses and accordingly regulates stress-response proteins. In addition, it

counterbalances the activity of the *agr* system on virulence factor expression and thus overlaps with the *agr* regulon (178). In *S. aureus*, the *sigB* gene is part of an operon, formed with *rsbU*, *rsbV* and *rsbW*. Expression of *sigB* is driven by SigA, which leads to the transcription of the whole operon, as well as through autoregulation by SigB itself, leading to transcription of *rsbV* and *rsbW* (179). SigB activity is regulated on the post-transcriptional level by the Rsb (regulation of sigma B) proteins. SigB is bound and kept inactive by the anti-sigma factor RsbW during exponential growth. Binding of the anti-anti-sigma factor RsbV leads to the release of SigB from RsbW and subsequently allows its binding to the RNA-polymerase holoenzyme (180). The first gene in the SigB operon, *rsbU*, was shown to be necessary for the activation of SigB (181) and is the major activator of SigB during acidic stress (182).

In *S. aureus*, SigB directly and indirectly controls approximately 200 genes, including genes with functions in virulence, biofilm formation, persistence, cell internalization, membrane transport and antibiotic resistance (180). Several of these regulatory functions are executed by the regulation of known virulence regulators, like *agr* and SarA (29). An early study on the phenotypic variation of wildtype *S. aureus* strains, and their corresponding *sigB* mutants, suggested a role of SigB in staphyloxanthin pigment production and sensitivity to hydrogen peroxide (183), demonstrating its role in stress response. Strain 8325–4 is a natural *rsbU* mutant and produces high levels of protease and alpha-toxin activity, and this phenotype is dramatically reduced when *rsbU* is repaired (184, 185). Similar results were obtained using the clinical isolate KS26, a prototype V8 protease producer, and strain Wood46, a hyper-alpha-toxin producer, and both of these strains were shown to have mutations in the *sigB* operon (186). Another natural *rsbU* mutant strain is BB255, rendering it effectively a SigB deficient strain. Complementation of *rsbU*, as well as SigB overproduction in this strain background, increased the strain attachment to fibrinogen- and fibronectin-coated surfaces, as well as adherence to platelet-fibrin clots (187). These phenotypes are thought to derive from the positive regulation of the cell wall-attached adhesins ClfA and FnbA by SigB (187, 188). SigB also regulates TSST-1 through an indirect mechanism involving the two virulence regulators *agr* and SarA (134, 189). In addition, the enterotoxin Seh and the enterotoxin gene cluster *egc* are activated by SigB, while transcription of enterotoxin Seb is negatively regulated by SigB (189). SigB also plays an important role during conditions present in the pulmonary environment. When colonizing the lung, bacteria come into contact with pulmonary surfactant that damages the bacterial cell membrane. In response to the surfactant, *S. aureus* induces the expression of the γ -hemolysin subunit B, a component of the Type-VII secretion system, and the PsiA damage repair protein. The upregulation of these genes is dependent on SigB, indicating that SigB plays an important role in *S. aureus* adaption to the lung environment (190).

SigH

Besides SigB, *S. aureus* harbors a second alternative sigma factor, known as SigH. It was recently shown that overexpression of SigH leads to natural competence of *S. aureus*, although with very low efficiency (191). Indeed, some genes known to be involved in natural competence of other bacterial species are regulated by SigH (192, 193), but several other competence genes are expressed poorly in *S. aureus* and are not effected by SigH (193).

SigH also stabilizes the lysogenic state of *S. aureus* prophages (194). As prophages often carry genes for virulence factor production, SigH might play an underappreciated role in *S. aureus* virulence.

CONCLUSION

As outlined in this chapter, the regulation of *S. aureus* virulence factors is subject to a complex and intricate network of factors that integrate physiological processes in response to environmental cues and host signals. This complex network allows *S. aureus* to readily adapt to changing conditions and establish itself in the available niches through its commensal and pathogenic life cycle. Some of the regulatory schemes outlined in this chapter are highly specialized and fine tune gene expression in a unique environment, such as in a specific host site. On the other hand, *S. aureus* has evolved several master regulators of virulence that have more global effects, with the most prominent being the *agr* quorum-sensing system, SaeRS, SarA, Rot, ArlRS and MgrA as summarized herein. An overview of the interconnections between these global regulators is depicted in Figure 6.

Going forward, there is growing interest in targeting regulatory pathways as a means of preventing infection. As antibiotic resistance in *S. aureus* continues to spread (195), new and unconventional approaches to fight bacterial infections are needed to maintain antibiotic stewardship (196). Due to the importance of the *agr* system in virulence, researchers have extensively targeted this system as a potential means of therapy (197–199). Efforts have been made to target the other global regulators, but in some cases these inhibitors have had broader effects on numerous systems (200). In regards to the *agr* inhibitors, some of the best and most effective candidates are the AIPs themselves, and numerous synthetic studies have been performed to identify improved and more global AIP-based inhibitors (28, 198). More recently, it has been realized that commensal microbes sharing the same niche as *S. aureus* may compete through *agr* interference (90, 201), which ultimately may dictate the ability of *S. aureus* to colonize, or not colonize, the host. An improved understanding of these natural competition mechanisms may shape future treatment approaches for certain types of infections.

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Figure 1.

A schematic of the molecular organization, signal biosynthesis and transduction cascade of the *agr* quorum-sensing system. The autoinducing peptide (AIP) signal is encoded within the AgrD peptide. AgrD is processed and transported into the environment by AgrB, with the aid of signal peptidase SpsB. When the extracellular AIP concentration reaches a critical level, the signal is sensed by the histidine kinase AgrC, which undergoes auto-phosphorylation. Then the phosphate is relayed to AgrA, which in turn can bind the P2 and P3 promoters, driving expression of the RNAII and RNAIII transcripts, respectively. The RNAII transcript harbors the *agrBDCA* operon, encoding the primary machinery for AIP biosynthesis and detection. RNAIII is the main effector molecule of the *agr* system and drives expression of downstream target genes. Phosphorylated AgrA also binds the promoters for the phenol-soluble modulins (PSMs) genes, leading to their expression.

Figure 2.

The autoinducing peptides (AIPs) and *agr* interference within *S. aureus* strains. Every *S. aureus* strain has a single *agr* system that can produce one of four different AIP signal structures. Each of the AIPs has a five-residue cyclic thiolactone ring, but the amino acids within the ring and the N-terminal extension are variable. The Type I and Type IV AIPs differ by only one amino acid and can function interchangeably, while the Type II and III AIPs are more divergent. Interference is observed between the three different groups of AIPs as shown. In each case, the cognate AIP signal from a producing strain will cross-inhibit the AgrC receptor, and in turn inhibit *agr* function, on a *S. aureus* strain representing a different *agr* group.

Figure 3.

Schematic of the molecular organization and signal transduction of the SaeRS TCS. (Top) The histidine-kinase SaeS phosphorylates its cognate response regulator SaeR. Phosphorylated SaeR can then bind to the promoter region of target genes and induce expression of numerous virulence factors (listed). The phosphorelay from SaeS to SaeR is inhibited by the combined action of SaeP and SaeQ. (Bottom) The *sae* gene cluster consists of four genes, *saeP*, *saeQ*, *saeR* and *saeS*. All four genes are transcribed from the P1 promoter. In addition, transcription of *saeS* and *saeR* is enhanced via the P3 promoter, which is located within the coding region of *saeQ*.

Figure 4.

The *sarA* promoter region and SarA regulatory network. *sarA* gene expression is driven by three different promoters (P1, P2 and P3) as shown. The alternative sigma factor σ^B (SigB) drives expression of *sarA* by binding to the P3 promoter. The binding of SarR to all three promoters inhibits expression and impedes autoregulation by SarA. Finally, SarA is an activator of the *agr* system, and it can also function as a negative regulator of the three SarA-like proteins SarH1, SarT and Rot.

Figure 5.

Regulatory pathway of ArlRS TCS and MgrA in biofilm formation and clumping. **A.** The ArlRS TCS is activated by an unknown signal and subsequently activates MgrA, which in turn represses the production of large surface proteins (Ebh, SraP and SasG), allowing ClfA/ClfB to interact with fibrinogen (Fg). Neighboring cells binding to the dimeric Fg molecule leads to clumping. **B.** When ArlRS is inhibited, MgrA is not expressed and the repression of the large surface proteins (Ebh, SraP and SasG) is relieved, preventing proper interactions of ClfA/ClfB with Fg. At the same time, SasG overproduction leads to homodimeric interactions with other cells expressing SasG, resulting in enhanced biofilm formation. This figure is a reproduction from one published in the following article (84).

Figure 6. Interaction network of the major *S. aureus* global regulators. In the schematic shown, a comprehensive overview of five regulatory systems is depicted, including the *agr* quorum-sensing system, ArlRS and SaeRS TCS, and three members of the SarA-protein family (SarA, Rot and MgrA). The virulence-associated traits controlled by each regulator is also shown.

Table 1.Major virulence regulatory systems of *S. aureus*

Regulator system	Role	In vivo	Reference
<i>agr</i>	Cell-to-cell communication (quorum sensing) with AIPs as signal; <i>agr</i> activation leads to expression of exo-toxins and exo-enzymes	Required for virulence in animal models of skin infection, pneumonia, and endocarditis	(27, 30, 50–54, 56, 59, 62, 87, 89–92, 94)
SaeRS	Induction of exo-protein production, including many virulence factors	Required for virulence in animal models of skin infection and pneumonia	(97, 98, 105–108)
SrrAB	Oxygen-responsive TCS; induction of <i>plc</i> and <i>ica</i> expressin; repression of <i>agr</i> , TSST-1 and <i>spa</i>	Required for defense against neutrophils	(71, 80, 113, 118)
ArlRS	Autolysis and cell surface TCS; induction of MgrA expression and repression of <i>agr</i> and autolysis	Required for virulence in animal models of skin infection and endocarditis	(121–123, 125, 202)
SarA	Cytoplasmic regulator; induction of exo-proteins and repression of <i>spa</i>	Required for virulence in animal models of biofilm infection	(135, 137, 139, 161)
Rot	Cytoplasmic regulator of toxins and extracellular proteases; <i>agr</i> activation prevents Rot translation	Mutation of <i>rot</i> restores virulence in <i>agr</i> -null background in rabbit endocarditis model	(61, 155–160)
MgrA	Cytoplasmic regulator; Induction of efflux pumps and capsule expression; repression of surface proteins	Required for virulence in animal models of skin infection and endocarditis	(84, 163, 164)
SigB	Stationary phase sigma factor; inhibits <i>agr</i> activity	Important for the establishment of chronic infection in rat lung model	(134, 183–188, 190, 203)

Table 2.Regulators impacting *agr* system function.

Regulator	Impact on <i>agr</i>	function	Reference
ArlRS	negative	Represses <i>agr</i> P2 and P3	(121)
CcpA	positive	Activates <i>agr</i> P3	(204)
CodY	negative	Repress <i>agr</i> P1 promoter in <i>agrC</i>	(79, 80)
MgrA	positive	Activation of <i>agr</i> P3	(82)
Rsr	negative	Represses <i>agr</i> P3	(205)
SarA	positive	Activates <i>agr</i> P2 expression	(136, 206)
SarR	negative	Represses <i>agr</i> P2 expression	(136)
SarT	negative	Represses <i>agr</i> P3	(147)
SarU	positive	Activation of <i>agr</i> P2 and P3	(148)
SarX	Negative	Represses <i>agr</i> P2 and P3	(207)
SarZ	positive	Activates <i>agr</i> promoter	(208)
SrrAB	negative	Represses <i>agr</i> P2 and P3	(71, 117)
σ^B	negative	Represses <i>agr</i> P3	(185, 209)