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GLOBAL REGULATION OF VIRULENCE DETERMINANTS IN *STAPHYLOCOCCUS AUREUS* BY THE SarA PROTEIN FAMILY

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1. ABSTRACT

In *S. aureus*, the production of virulence determinants such as cell wall adhesins and exotoxins during the growth cycle is controlled by global regulators such as SarA and *agr*. Genomic scan reveals 16 two-component regulatory systems (e.g. *agr* and *sae*) as well as a family of SarA homologs in *S. aureus*. We call the SarA homologs the SarA protein family. Many of the members in this protein family are either small basic proteins (<153 residues) or two-domain proteins in which a single domain shares sequence similarity to each of the small basic proteins. Recent crystal structures of SarR and SarA reveal dimeric structures for these proteins. Because of its structure and unique mode of DNA binding, SarR, and possibly other SarA family members, may belong to a new functional class of the winged-helix family, accommodating long stretch of DNA with bending points. Based on sequence homology, we hypothesize that the SarA protein family may entail homologous structures with similar DNA-binding motifs but divergent activation domains. An understanding of how these regulators interact with each other *in vivo* and how they sense environmental signals to control virulence gene expression (e.g. α-hemolysin) will be important to our eventual goal of disrupting the regulatory network.

2. INTRODUCTION

Staphylococcus aureus is an important pathogen both in the community and hospital settings (1). The spectrum of diseases caused by this organism is extremely wide, ranging from superficial skin infections to deep-seated abscesses. Many of these infections begin locally

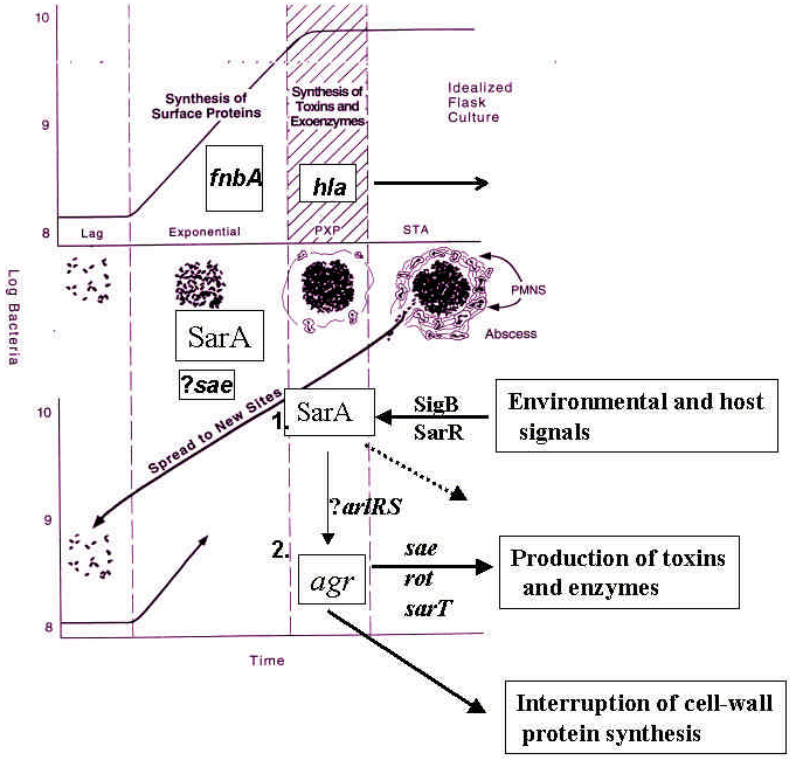

Figure 1. The expression of virulence determinants as controlled by global regulatory loci during the growth cycle based on mostly *in vitro* data**.**

(e.g. skin and catheters) and subsequently spread to the bloodstream, thus putting patients at risk of developing endocarditis and other metastatic complications (1). Despite antimicrobial therapy, the morbidity and mortality remain high, in part due to the organism's ability to develop resistance to many antibiotics (1). The recent report of reduced susceptibility to vancomycin, first in Japan and subsequently in the United States (1), has raised the concern that resistant *S. aureus* infections may be difficult to treat with preexisting antibiotics.

The pathogenicity of *S. aureus* is a complex process involving a diverse array of extracellular and cell-wall components. With the exception of toxin-mediated syndromes, it has been difficult to implicate a single factor that would explain the myriad of staphylococcal diseases (1). Many cell-wall components that act as adhesins (e.g. fibrinogen and fibronectin binding proteins) or contribute to the evasion of host defenses (e.g. protein A) are produced primarily during the exponential phase while the production of toxins and enzymes (e.g. α-toxin) that facilitate tissue invasion occurs postexponentially, at least in laboratory cultures. The coordinate synthesis of cell-wall proteins in the exponential phase and extracellular proteins during the postexponential phase suggests that many of these virulence determinants are governed by phenotypic switches or global regulatory elements (Figure 1) (1). One of these molecular switches is SarA (Staphylococcal accessory regulator A) which, in combination with *sae*, activates the synthesis of cell-wall proteins during the exponential phase (2) (3). Another molecular switch, the *agr* regulon (accessory gene regulator), up-regulates the synthesis of many extracellular toxins and enzymes while down-regulating the production of cell-wall proteins postexponentially (4). However, the bacterial signals, in particular those generated in response to host environments (e.g. tissues factors and phagocytic cells), remain poorly defined. To elucidate these host-induced bacterial factors, several approaches have been devised to identify bacterial genes that are differentially expressed within the host environment during infections including: a) **differential display** by identifying differentially expressed mRNA transcripts in host environments (5); b) *in vivo* expression technology (**IVET**) for selecting promoter fragments linked to an reporter gene expressed in hosts (6, 7); c) signature-tagged mutagenesis (**STM**) with a negative selection strategy to identify tagged transposon mutants (8, 9); and d) differential fluorescent induction (**DFI**) in which *in vivo*-activated promoter fragments linked to a green fluorescent protein (GFP) reporter gene of the jellyfish *Aequorea victoria* are identified by a fluorescence activated cell sorter (FACS) (10, 11). The identification of host-induced bacterial genes will likely provide new insights into the role of virulence factors essential to initiating the infection. Additional regulatory factors that modulate these virulence genes can also be elucidated as part of the pathogenic processes.

In this chapter, we will summarize the recent data on the regulation of pathogenicity in *S. aureus*. Our

Table 1. Virulence factors of *S. aureus*

Virulence Factors	Putative functions
Cell-wall/membrane constituents	
Bone sialoprotein binding protein (bbp)	Adhesion to bone sialoprotein
Capsular polysaccharides (cap)	Anti-phagocytic molecule
Clumping factor A (clfA)	Adhesin to fibrinogen
Clumping factor B (clfB)	Adhesin to fibrinogen
Coagulase (coa)	Binding to fibrinogen
Collagen binding protein (cna)	Adhesin to collagen
Dlt (dlt)	Resistance to defensins
Elastin binding protein (ebpS)	Binding to elastin
FibA protein (fibA)	Binding to fibrinogen
Fibronectin binding proteins (fnbA,B)	Attachment to fibronectin
Laminin binding protein	Adhesion to matrix protein
MHC analogous protein (map or eap)	Binding to extracellular matrix proteins including fibronectin, fibrinogen, vitronectin, bone sialoprotein and thrombospondin
MprF (mprF)	Resistance to defensins
Plasminogen binding protein	Binding to plasminogen
Polysaccharide intracellular adhesin (pia)	Intercellular adhesion and biofilm formation
Protein A (spa)	Possible evasion of host defenses (binding to Von Willebrand Factor)
Sdr C-E (sdrC,D,E)	Possible adhesion to host tissues
Thrombospondin binding protein	Binding to thrombospondin
Vitronectin binding protein	Binding to host matrix
Extracellular toxins and enzymes	
∀-hemolysin (hla)	Tissue invasion, form pores in host cell membrane
∃-hemolysin (hlb)	Tissue invasion, sphingomyelinase
∗-hemolysin (hld)	Potentiation of ∃-hemolysin
Enterotoxins A-E, G-Q (sea-e, seg-q)	Evasion of host defenses with superantigen functions, causative agents for food-borne diarrhea
Exfoliative toxins A,B (eta, etb)	Evasion of host defenses, causative agents for staphylococcal scalded skin syndrome (SSSS)
(-hemolysin (hlgA, B, C)	Potentiation of host cell lysis
Sets 1-5, 8-9	Exotoxin-like proteins
Toxin shock syndrome toxin-1 (tst)	Evasion of host defenses with superantigen properties, causative agent for TSS
Catalase	Neutralizing hydrogen peroxide produced from phagocytes
Cysteine protease (sspB)	Tissue invasion
Elastase (sepA)	Tissue invasion
Fatty acid modifying enzyme (FAME)	Tissue invasion
Hyaluronic lyase (hysA)	Tissue invasion
Lipase (geh)	?Evasion of host defense
Metalloprotease (aur)	Tissue invasion and surface protein modification
Phospholipase C (plc)	Lysis of host cells
Panton-Valentin leukocidin (lukF, lukS)	Evasion of host defenses, lysis of host phagocytes
Serine proteases (slpA,B,D,F)	Tissue invasion
Staphopain (scp)	Tissue invasion
Staphylokinase (sak)	Evasion of host defenses
V8 protease (sspA)	Tissue invasion and surface protein modification

Data for this tables are compiled from several sources (12, 23, 118, 119, 120, 121, 122).

discussions are by no means comprehensive, but rather serve to provide a platform upon which future research endeavors can be assembled. Predicated upon recent genomic and crystallographic data, we will focus, in particular, on global regulation by two-component regulatory systems and the SarA protein family. For a detailed discussion of *agr*, the prototypic member of the two-component regulatory systems in *S. aureus*, readers are referred to an excellent review by Arvidson and Tegmark (12). Although the majority of the data supporting this and recent reviews are generated from *in vitro* studies, it is our belief that, as the roles of these pathogenicity genes are assessed *in vivo*, our perception of their relevance in human infections may be transformed.

3. REVIEW

3.1. Global Regulation of virulence determinants during *in vitro* growth

The coordinate regulation of extracellular and cell-wall virulence determinants during bacterial growth implies that many of these virulence factors are likely controlled by global regulatory elements. Unless specifically mentioned, the majority of these regulations are found to occur at the transcriptional level. During the exponential phase, cell wall proteins, many of which are adhesins, are synthesized. These cell wall adhesins, also called MSCRAMMS (microbial surface components recognizing adhesive matrix molecules), include protein A, fibrinogen binding proteins, fibronectin-binding proteins, collagen binding protein etc. (Table 1). The synthesis of these surface adhesins is likely activated by global regulatory loci such as *sarA* and *sae* (2, 3). In transition from the exponential to the postexponential phase, environmental signals are generated to activate *agr*, *sarR*, *sigB* and other regulatory elements (4, 13-18) while regulatory elements such as *rot*, *sarS*, *sarT* and, possibly other unknown factors, are repressed (Figure 1, see details below) (19-21). Additional regulatory signals, such as those identified by genomic analysis and *in vivo* selection strategies, may also modulate these regulatory elements.

The SarA protein, the major *sarA* regulatory molecule, is maximally expressed during the late exponential phase and tapers postexponentially. The expression of the SarA protein coincides with the

Table 2. Virulence determinants regulated by *sarA* and *agr*

	sarA	agr
Aureolysin (Metalloprotease)	-	+
Enterotoxin B	+	+
Enterotoxin C	Unknown	+
∀-hemolysin	+	+
∃-hemolysin	+	+
∗-hemolysin	+	+
γ-hemolysin	+	+
Exfoliatins A and B	Unknown	+
FAME (fatty acid modifying enzyme)	+	+
FemA	+	Unknown
FemB	Unknown	+
HysA (hyaluronate lyase)	Unknown	+
Lipase	-	+
Phospholipase C	Unknown	+
Set8 (exotoxin)	Unknown	+
Set9 (exotoxin)	+	Unknown
SplA,B,D and F (proteases)	+	+
SspB (cysteine protease)	-	Unknown
Staphylokinase	Unknown	+
TSST-1	+	+
V8 protease (SspA)	-	+
Clumping factor A	Unknown	No effect
Clumping factor B	+	No effect
Coagulase	+	-
Collagen binding protein	-	±
Capsular polysaccharide (type 5)	+	+
Fibronectin binding protein A	+	-
Fibronectin bindng protein B	+	-
GyrA	Unknown	-
LrgAB	+	+
PBP3	-	Unknown
Protein A	-	-
SdrC	+	Unknown
Vitronectin binding protein	Unknown	-

Data are derived from published materials (12, 23, 118).

expression of cell wall proteins such as fibronectin binding proteins, consistent with the direct regulation of *fnbA* (22) and possibly *fnbB* (23) by SarA (Table 2). In transition from exponential to postexponential phase, the expression of SarA probably reaches a critical threshold to facilitate binding to the *agr* promoter to augment *agr*-related transcription (Figure 1) (24). As with many of the prokaryotic global regulatory systems, repressors, such as the *arlSR* locus as reported by Fournier *et al* (25), may exist to suppress *agr* transcription. The *agr* locus plays an important role in repressing the synthesis of surface proteins (e.g. protein A) while up-regulating expression of extracellular enzyme and toxin genes (e.g. *hla*) during the postexponential phase. As the phenotypic outcome of *agr* is pleiotropic, one anticipates additional regulatory factors for this *agr*-mediated effect. For instance, the *agr* locus likely mediates *spa* repression by suppressing the transcription of *sarS*, a *sarA* homolog that is an activator of *spa* expression (20) (Figure 2). The up-regulation of *hla* by *agr* is more complex and entails intermediary genes such as *sae*, *rot* and *sarT* (Figure 1). The *sae* locus encodes a two component regulatory system, *saeRS*, that up-modulates *hla* transcription (3, 26). Transcriptional analysis indicated that *sae* likely acts downstream of *agr*. The Rot protein is likely a repressor of *hla*, with limited sequence similarity to SarA (19). Repression of the alpha hemolysin gene, *hla*, by the Rot protein is only evident in an *agr* mutant but not in the wild type strain, thus suggesting that Rot may somehow interact with the *agr* gene product or act downstream of *agr*. *sarT*, a repressor of *hla* transcription, was recently discovered in a search for SarA homologs in the *S. aureus* genome (27). The repression of *sarT* by *sarA*, and to a lesser extent by *agr*, would seem to place *sarT* downstream of these regulatory loci. However, this interpretation is derailed by the observed increase in *agr* expression in a *sarT* mutant. These observations, coupled with the finding that the SarA protein might induce *hla* expression by directly repressing *sarT* (27), imply that *sarA* and *agr* may modulate *sarT* expression differently to augment *hla* transcription. Whether *sae*, *rot* and *sarT* interact with each other to modulate *hla* transcription remains to be defined. Nevertheless, it is of significant interest that several regulatory factors are involved in the regulation of *hla* transcription. We hypothesize that these regulatory elements may optimize α-hemolysin secretion to ensure bacterial survival in selected host niches.

In addition to *sae*, *rot* and *sarT* acting downstream or in concert with *agr*, SarA, the major *sarA* regulatory molecule, is also capable of up-modulating *hla* transcription by binding directly to the *hla* promoter (28). Expression of the SarA protein is also controlled by SigB and possibly other stress-induced genes that are active during the postexponential phase (15). An additional regulatory protein, designated SarR for its homology to SarA, was found to bind to the *sarA* promoter region to down-regulate SarA protein expression (Figure 3). As evaluated in liquid culture studies, the confluence of these regulatory signals likely contributes to a significant up-regulation in the synthesis of toxins and, by extension, enzymes during the postexponential phase. Presumably, increased secretion of these toxins and enzymes will facilitate host cell lysis, thereby allowing the bacteria to spread to new sites.

3.2. Regulation of virulence determinants by two-component regulatory systems in *S. aureus*

Many of the virulence determinants in bacterial pathogens are controlled by two-component regulatory systems (4, 29-32) in which a membrane sensor, upon receiving an extracellular signal, would activate a response regulator by phosphorylation. The phosphorylated regulator, in turn, would bind to the target gene promoter to activate transcription. Because the C-terminal domain of the sensor molecule and the N-terminal domain of the response regulator are conserved, many of the proteins within the two-component systems can be identified by sequence alignment. Using this approach, ~16 two-component regulatory systems can be identified in the *S. aureus* genome (personal communication, Steve Gill). Among these, six have been previously identified including *agrAC*, *saeRS*, *lytRS*, *arlRS*, *srrAB* (also called *srhRS*) and *yycFG*. Molecular analysis indicated that all of these systems, to a degree, are involved in the expression of virulence determinants in *S. aureus*.

3.2.1. The *agr* locus

The *agr* locus is the site of a transposon insertion in a pleiotropic exoprotein-defective mutant (33, 34). Cloning of the chromosomal sequence adjacent to this insertion has led to the identification of the *agr* regulon (4). The *agr* locus is composed of two divergent transcripts (RNAII and RNAIII) driven by two distinct promoters [P2

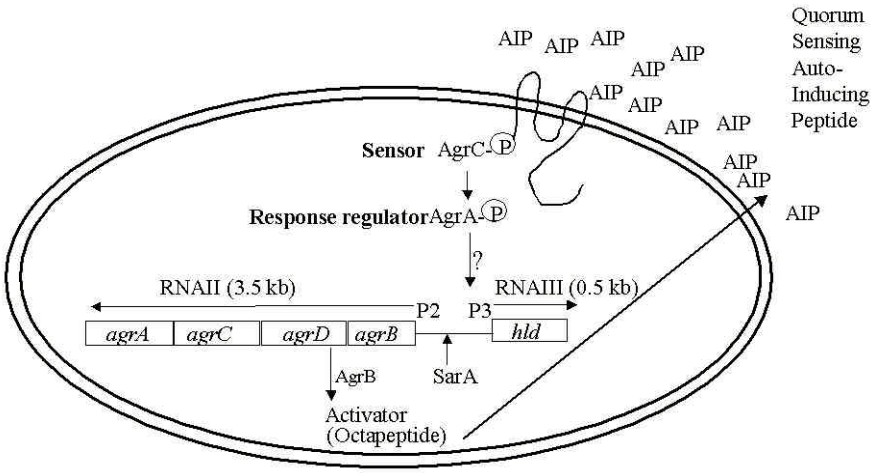

Figure 2. A model of the *agr* regulatory system. AgrC and AgrA are the kinase sensor and the transcriptional activator, respectively, of a two-component regulatory system. AgrD encodes a 46-residue peptide which is processed and secreted with the aid of the putative membrane component AgrB. The processed peptide (AIP) carries quorum-sensing function.

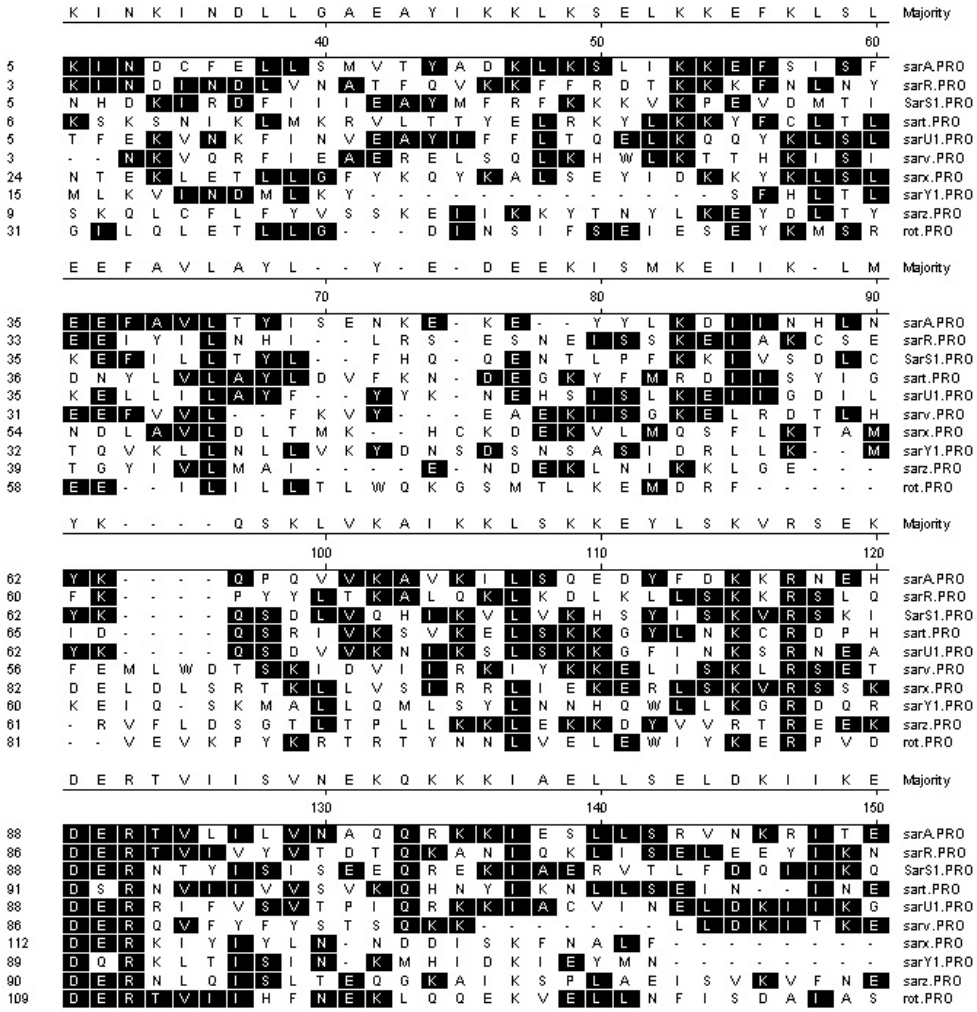

Figure 3. Alignment of SarA homologs. The majority of the residues are displayed. Please note that only the N-terminal halves of SarS, SarU and SarY are used for alignment.

and P3 promoters, respectively (Figure 2)]. The P2 transcript encodes four genes, *agrB*, *D*, *C*, and *A*, all required for the normal activation of RNAII and RNAIII (35-37). The P3 transcript encodes the 26 amino acid ∗-hemolysin peptide, which has no defined role in regulation (35, 37). In contrast to other regulatory paradigms where the effector molecules are proteins, the regulatory molecule within *agr* is the RNAIII transcript, acting mainly at the level of transcription (35, 37) and, to a lesser extent, translation (38).

AgrC and AgrA are the membrane sensor and the response activator, respectively, of a two-component regulatory system which is autoinduced by a post-translationally modified peptide with quorum sensing function (39, 40). The auto-inducing peptide (AIP) is processed from within the 46-residue *agrD* gene product (39) and then secreted, presumably with the aid of a 26 kDa transmembrane protein called AgrB (Figure 2). In contrast to the linear-peptide quorum-sensing molecules in other Gram+ bacteria (32, 41), the autoinducing peptide of *S. aureus* undergoes cyclization (via a thiolactone bond) between the cysteine residue and the C-terminal carboxyl group, leading to the formation of a cyclic peptide (42, 43). Structure-function studies have shown that the linear peptide is inactive (42), thus hinting at the importance of the ring structure for function.

The autoinducing peptide, upon reaching a critical concentration as a result of bacterial accumulation in liquid culture, would activate the 46-kDa transmembrane sensor protein, AgrC, by phosphorylating a specific histidine residue (44). A second phosphorylation step of the aspartic residue of AgrA, a 34-kDa cytoplasmic protein, would ensue, thus activating AgrA, the response regulator. Although phosphorylated AgrA has been predicted to activate the P2 and P3 promoters of *agr*, purified recombinant AgrA has failed to bind the intergenic region between the P2 and P3 promoter (45). This failure in binding may be explained by a need to phosphorylate recombinant AgrA or a failure for AgrA to re-nature upon purification in the presence of a denaturing agent (i.e. urea) (45). An alternative mechanism for RNAIII activation has been suggested by Balaban *et al.* (46) who reported the isolation of a 38-kDa protein (RAP) that is not encoded by *agr*. Activation of RNAIII by RAP requires phosphorylation of a 21-kD protein called TRAP. However, the claim of RAP as a RAIII-activating protein, rather than a peptide, is being disputed by other investigators who failed to isolate RAP from an *agr* null strain (43).

Once RNAIII is synthesized, it somehow up-regulates the transcription of exoprotein genes (e.g. hemolysins) while down-regulating that of cell-wall protein genes (protein A and fibronectin binding protein genes). However, the exact manner by which RNAIII activates target gene transcription is not clearly defined. In addition to its transcriptional effect, studies by Morfeldt and colleagues (38) indicated that the effect of RNAIII on *hla* expression may be translational, possibly by unwinding the secondary structure in the 5' end of the nascent *hla* mRNA, which, if left folded, interferes with the initiation of translation.

The *agr* locus is conserved among staphylococci. Nevertheless, sequence variations within *agrB*, *agrD* and *agrC* have resulted in productions of different AIPs and sensors (AgrC) with diverse specificities (39, 47, 48). Within *S. aureus*, four AIP groups (Group I-IV) have been identified. Cross activation of RNAIII is observed within each group while cross-inhibition occurs between diverse groups (39). Interestingly, AIPs from *S. epidermidis* can inhibit the *agr* response of *S. aureus* (47). Of note, Mayville *et al.* recently found that a synthetic Group II thiolactone peptide of *S. aureus* attenuated a murine experimental skin abscess infection due to a Group I strain (42). As the concentration of bacteria required to produce "reactive" concentrations of AIP is quite high (~10^8 CFU), the role of AIP in acute *S. aureus* bacteremic infections, where the bacterial dosage is relatively low (10^2-10^3 CFU/ml of blood), is not well defined. However, in abscesses or bacteria sequestered inside neutrophils or specialized epithelial cells (49, 50), one can envision that local concentrations of AIP might reach a level high enough for RNAIII activation.

3.2.2. The *saeRS* locus

The *sae* locus was originally identified by a Tn*551* insertion into *S. aureus* strain 196E (51). The *sae* mutant had reduced levels of α- and β- hemolysins, DNase, coagulase and protein A in the culture supernatant, primarily due to reduced transcription (3). Molecular analysis revealed that the *sae* locus encodes a two-component regulatory system with a kinase sensor and response regulator (*saeRS*) (26). As a *sae* mutation did not affect *agr*, it is likely that *sae* lies downstream of *agr*; alternatively, *sae* is totally independent of *agr* in its mode of action (3).

3.2.3. The *lytRS* locus

lytRS was identified by a PCR-based strategy to isolate potential sensor DNA fragments from *S. aureus* genome (52). The *lytS* mutant has a diffuse and rough cell surface, exhibits increased autolysis and an altered level of murein hydrolase activity as compared with the parental strain (52). LytRS regulates *lrgAB*, an operon that is involved in negative modulation of murein hydrolase activity and penicillin-induced lysis during the stationary phase of growth (53). Thus, the *lytRS* regulon indirectly controls the integrity of cell wall by affecting intrinsic murein hydrolase activity via LrgAB.

3.2.4. The *arlRS* locus

The site of transposition of a *S. aureus* transposon mutant from strain MT142 that exhibited increased autolysis and altered peptidoglycan hydrolase activity was found to be near the 5' end of the *arlS* gene, the sensor component of the *arsRS* two-component regulatory system (25, 54). Mutations in either *arlR* or *arlS* increase the transcription of many exoproteins genes including that of α-hemolysin and serine protease. Interestingly, the secretion of extracellular protein A is also reduced in the mutant. The *arl* mutants were found to

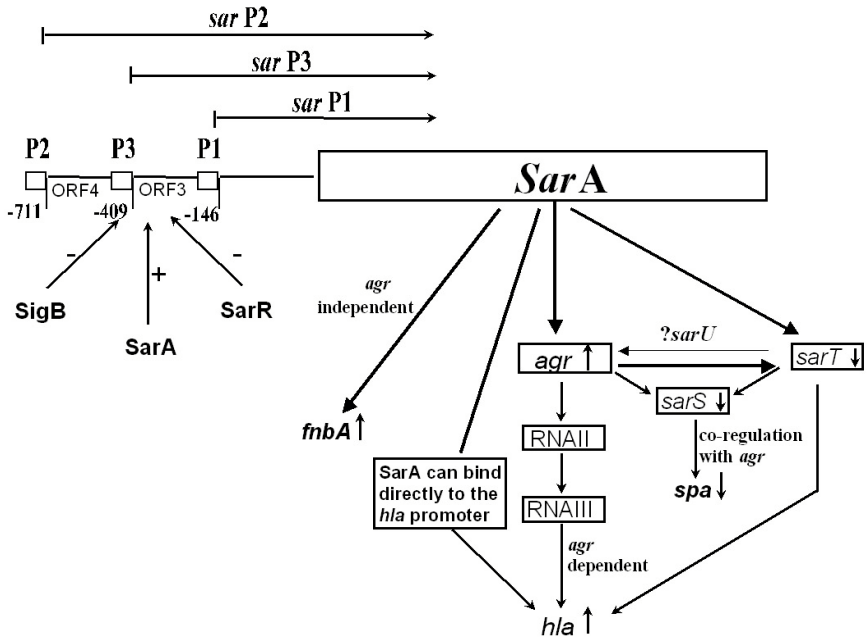

Figure 4. The putative regulatory pathways of the *sarA* locus. The expression of the major regulatory molecule, SarA, is probably controlled by SarA, SarR and SigB. The SarA protein level likely determines the degree of activation (*hla*, *fnbB*) or repression (*spa*) of target genes. Presumably, SarA binds to a conserved binding site upstream of the target gene promoter. Based on its interaction with *agr*, the *sarA* regulatory system can be divided into three major pathways: 1) ∀-hemolysin activation pathway (*hla* via *agr* and via direct binding of SarA to the *hla* promoter); 2) protein A repression pathway (co-regulate with *agr* to suppress *spa* transcription); and 3) fibronectin binding protein activation pathway (*fnbA* expression independent of *agr*). However, additional levels of complexities arise because of intermediary genes such as *sarS*, *sarT* and *sarU*.

increase the expression of RNAII and RNAIII, thus suggesting that *arlRS* likely lies upstream of *agr* to repress exoproteins gene transcription (Figure 1). Surprisingly, the *arl* mutations did not affect *spa* expression in an *agr* mutant or in a *sarA* mutant, consistent with the notion that *arl* interacts with these two loci in a complex manner to control *spa* expression (25).

3.2.5. The *srrAB* (*srhSR*) locus

The *srrAB* operon, also called *srhSR* by another group (55), is a homolog of the *resDE* genes in *B. subtilis* (56). A mutation in the *ssrAB* operon has led to increased expression of RNAIII and TSST-1, especially under microaerobic conditions. Like ResDE (57, 58), SsrAB may act as a sensor-response regulator under low oxygen conditions. A mutation in the *srhRS* locus has resulted in a dramatic reduction in growth under anaerobic conditions and a 3-log attenuation in the murine pyelonephritis model (55), consistent with the notion that *ssrAB* is an important regulator of energy transduction in response to changes in oxygen availability.

3.2.6. The *yycFG* locus

The *yycFG* two-component system, first discovered in *B. subtilis* by Fabret and Hoch (59), was also found in other Gram+ species including *S. pneumoniae* and *S. aureus* (60, 61). The *yycFG* system is essential to the growth of *B. subtilis*, *S. pneumoniae* and *S. aureus*. Studies with a conditional *yycFG* mutant of *S. aureus* revealed the role of YccFG in regulating cell wall composition. This finding was further supported by a recent report in *B. subtilis*, showing that YycF (response regulator) and YycG (sensor) modulate expression of the cell division operon *ftsAZ* (62).

3.3. Regulation of virulence determinants by the SarA protein family

Besides the two-component signal transduction system, the SarA protein family also controls many of the virulence determinants in *S. aureus*. The prototypic family member, SarA, was originally identified by a Tn*917*LT1 insertion into the *sarA* locus (2). Using promoter-DNA affinity columns as well as genomic scanning, at least 9 other SarA homologs can be identified (12, 13, 63) (Figure 3). Many of the SarA homologs are small basic proteins, ranging from 13 to 16 kDa in molecular masses. Three of the members, SarS, SarU and SarY, are larger in size, with molecular masses of ~30 kDa. Alignment of these three proteins with their smaller counterparts disclosed that SarS, SarU and SarY might compose of two homologous domains each of which shares sequence similarity with the smaller SarA homologs (Figure 3). Many of these homologs have been shown to participate in the SarA/*agr* regulatory cascade (Figure 4). For example, SarR binds to the *sarA* promoter region to down-regulate SarA expression (13). SarT is a repressor of alpha hemolysin synthesis (21). SarS, also called SarH1 (63), is an activator of protein A synthesis (64) while SarU activates RNAIII transcription (unpublished data). Although we currently do not know the exact functions of other SarA homologs, we speculate

that they may play regulatory roles as well.

3.3.1. The *sarA* locus

The *sarA* locus is composed of three overlapping transcripts, designated *sarA* P1, *sarA* P3 and *sarA* P2, originated from promoters P1, P3 and P2, respectively (Figure 4) (65). The promoter boxes (-10 and -35) of P1 and P2 have sequence similarity to the ΦA and Φ70 dependent promoters of *B. subtilis* and *E. coli*, respectively. In contrast, the *sarA* P3 promoter possesses a striking homology to the ΦB(SigB)-dependent promoter of *B. subtilis* (65-68). Activation of the ΦB-dependent promoters has been shown to be involved in the general stress response of Gram+ bacteria (69). Because of their overlapping nature, each of the *sarA* transcripts encodes SarA, a 14.7 kDa protein with a deduced pI of 8.52 (70). Because of its small size, a basic pI and a high percentage of charged amino acids (33%), SarA has been predicted to be a DNA binding protein (2, 71).

The promoter region of the *sarA* locus is quite extensive (~800-bp), with two smaller potential coding regions nested within the P2-P3 and the P3-P1 promoters, respectively. The sequence between P3 and the proximal P1 promoter has been shown to be crucial to the regulation of the *sarA* P1 promoter (66, 72, 73), however, the translation of the putative smaller coding region nested within the P3-P1 interpromoter region has so far not been demonstrated. Complementation analysis indicated that SarA is the major *sarA* regulatory molecule (72, 73), hence responsible for the up-regulation in the expression of hemolysins [∀ and ∃ toxins (73, 74)], toxin shock toxin (TSST-1) (75), enterotoxin B (75), fatty acid modifying enzymes FAME (12), fibrinogen binding proteins (e.g. coagulase) (2) and fibronectin binding proteins (22), and down-regulation in the synthesis of protein A (76), collagen binding protein (77), lipase (2) and proteases such as V8 protease (75), cysteine protease, aureolysin and staphopain (12) (Table 2). More recently, the *sarA* locus has been shown to repress autolytic activity (78). Because of the suppressive effect of *sarA* on V8 protease and aureolysin, recent data from Karlsson and colleagues disclosed that the decrease in fibronectin binding protein expression in a *sarA* mutant may be attributable to surface protein modification by these two proteases (79). Gene chip analysis indicated that the *sarA* locus affects the transcription of at least 120 genes in *S. aureus*, consistent with the global regulatory nature of this locus (23). Many of these SarA-mediated effects may be direct, as indicated by the presence of a consensus SarA binding site upstream of the –35 promoter of the affected gene (see below for SarA binding site). The absence of the SarA binding site in the remaining genes may imply indirect effects, possibly involving intermediate genes that, in turn, affect target genes downstream. Given the energy expenditure associated with activation of SarA, we speculate the expression of SarA to be tightly controlled during bacterial growth.

Several lines of experimental evidence suggest that the *sarA* locus regulates *agr* (Figure 1 and 4). In particular, the *agr* transcripts RNAII and RNAIII, diminished in *sarA* mutants, were restored upon complementation with intact *sarA* fragments (70). Additionally, hemolysin production, which is reduced in *sarA* mutants, can be restored to wild type levels by supplying a plasmid carrying RNAIII under an inducible promoter (70). The SarA binding site on the *agr* promoter has been mapped to a 29-bp sequence, locating between the *agr* P2 (-73 to -101 upstream of the P2 transcription start) and P3 promoters (-83 to -111 upstream of the P3 transcription start) (4, 28). In showing SarA as a dimer, Richtin *et al.* have mapped SarA to three binding sites, broader than the 29-bp binding site that we described, with each consisting of two half-sites binding a SarA dimer (80). Because greater amounts of SarA (requiring 6-50 nM of SarA corresponding to 88-735 :g of purified protein vs. 1-5 :g in our studies) were used in the footprinting reactions as described by Rechtin *et al.*, it is conceivable that technical differences may have accounted for the divergent results. Nonetheless, deletion studies (see below) of the putative 29-bp SarA binding site upstream of *agr* promoter indicated that this sequence is required for the transcription of RNAII of the *agr* locus in *S. aureus* (28).

The mechanism by which SarA, the major *sarA* effector molecule, regulates its assortment of target genes has recently been elucidated. In aligning the sequence upstream of the -35 promoters of several target genes, a consensus sequence, homologous to the 29-bp SarA binding site on the *agr* promoter as mapped by the DNase I footprinting assay, emerges (Figure 5). Gene chip and genomic analysis revealed that the consensus SarA binding site is present in at least 36 target genes (23). The importance of the consensus binding sequence in promoters of *hla* and *agr* has been confirmed by deletion studies in which *hla* and RNAII-containing fragments, devoid of the SarA recognition motif, failed to transcribe the respective genes as compared with the non-mutated controls (28), thus hinting at the essential role of the SarA binding site in target gene transcription. Remarkably, the transcription of *spa*, a gene normally repressed by *sarA*, also becomes de-repressed, as measured by a XylE reporter fusion assay, in a strain in which the SarA recognition motif has been deleted from the *spa* promoter region (28). On the basis of these findings, we formulated a hypothesis for virulence gene activation in *S. aureus* whereby SarA is the regulatory protein that binds to the consensus SarA recognition motif to activate (e.g. *agr* and *hla*) or repress (*spa*) the transcription of target genes, thus accounting for both *agr*-dependent and *agr*-independent modes of regulation. Based on the proposed pathway, it can be observed that *hla* transcription can be up-regulated via dual pathways, first via an up-regulation of *agr*, second via direct binding of SarA to the *hla* promoter (Figure 4).

Based upon its interaction with *agr*, the *sarA* locus probably regulates its target genes via at least three pathways (Figure 4). Thus, besides the *agr*-dependent *hla* pathway (28), the transcriptional repression of *spa* (the protein A pathway) by *sarA* is dependent on co-regulation with *agr* (76). For the third pathway (*fnbA*), the *sarA* locus up-regulates the transcription of target genes (*fnbA*) via an *agr*-independent mechanism (22).

```
agr         ATTTGTATTTAATATTTTAACATAAA    20/26
hla         ATTTTTATTTAATAGTTAATTAATTG    23/26
spa         AATTATAAATATAGTTTTTAGTATTG    17/26
fnbA        ACTTGAATACAATTTATAGGTATATT    16/26
fnbB        CTTTGTATGCAATATATATGTGAGTT    19/26
sec         ATTTTCTTTTAATATTTTTTTAATTG    23/26

Consensus ATTTgTATtTAATATTTataTAAtTg
                          t t
                            g
```

Figure 5. Alignment of a putative common SarA recognition sequence from promoters of *sarA* target genes. *hla*, ∀-hemolysin gene; *spa*, protein A gene; *fnbA*, fibronectin binding protein A gene; *fnbB*, fibronectin binding protein B gene; *sec*, enterotoxin C gene. The *agr* promoter sequence for SarA binding as mapped by DNaseI foorprinting assay is underlined (see text). The consensus sequence was derived as follows: 4/6, capital letter; 3/6, small letter; an even distribution of nucleotides at a specific position will be presented as combinations of small letters.

3.3.2. The *sarR* locus

SarR was identified upon passing *S. aureus* cell lysates through a DNA-specific affinity column containing a 49-bp sequence of the *sarA* P2 promoter (13). This P2 promoter sequence also shares homology with a 34-bp sequence upstream of the *sarA* P1 promoter (13). The putative gene, designated *sarR*, encodes a 115-residue protein (13.6 kDa) that shares homology with SarA (51% similarity and 28% identity) (Figure 3). The *sarR* gene is present in *S. aureus* strains and in *S. saprophyticus* as disclosed by hybridization studies (13). Purified SarR was found to bind *sarA* P1, P3 and P2 promoters by gel shift and DNase I footprinting assays. Allelic replacement of *sarR* with an *ermC* gene diminished *sarA* P3 transcription and enhanced P1 transcription in the mutant. As P1 is the strongest promoter within the *sarA* locus (66), we suspected and confirmed that a mutation in *sarR* would increase SarA expression (13). More importantly, the *sarR* mutant, correlative of an increase in SarA protein level, activated *agr* transcription to a higher degree than the parental strain (13). Taken together, these studies strongly support the notion that SarR is a DNA-binding protein that binds to the *sarA* promoter region to up-regulate P3 and down-regulate P1 transcription, with the net effect being a decrease in SarA protein expression (Figure 4).

3.3.3. The *sarT* locus

sarT was identified from a search of the *S. aureus* genome for SarA homologs (21). The *sarT* gene codes for a 118-residue protein (16.096 kDa) with a basic pI of 9.55. The expression of *sarT* was repressed by *sarA* and *agr*. SarT also suppressed the expression of *hla*, with the repression relieved upon mutating *sarT* (21). Because the *sarA*/*sarT* mutant exhibited elevated *hla* transcription as compared with the single *sarA* mutant, it is likely that the *sarA* locus promotes *hla* expression by repressing *sarT*. Contrary to the *sarA*/*sarT* mutant, *hla* expression in the *agr*/*sarT* mutant remained low, similar to the single *agr* mutant, thus indicating that *agr* does not up-regulate *hla* via repression of *sarT*. We also have evidence that the *agr*-mediated effect on *hla* is likely to be more complex since RNAIII expression was found to be elevated in the *sarT* mutant. Therefore, it is conceivable that *sarT* contributes to a feedback loop on *hla* expression, using *agr* as an intermediate.

3.3.4. The *sarS* (*sarH1*) locus

Similar to SarT, SarS was discovered in a search for SarA homologs in the *S. aureus* genome (20). SarS was identified as SarHI by Tegmark *et al.* who recovered this protein from *sarA* or *agr* mutant cell lysates with a DNA affinity column containing target promoter DNA fragments (63). The *sarS* gene, lying upstream of the *spa* gene, encodes a 250-residue protein (29.792 kDa) that can be envisioned as two homologous halves, with each half sharing sequence similarity with SarA, SarR and SarT (81) (Figure 3). The transcription of *sarS* was repressed by *sarA* and *agr*. Inactivation of *sarS* had led to a significant diminution in the synthesis of cell-wall protein A (63, 64). Transcription fusion studies with a *spa* promoter linked to the *xylE* reporter disclosed that the effect of *sarS* on *spa* is primarily transcriptional (64). Interestingly, the up-regulation in *spa* expression in an *agr* mutant returned to near parental level in an *agrA*/*sarS* mutant but not the *sarA*/*sarS* mutant in the RN6390 genetic background. Taken together, these data demonstrated that the *agr* locus likely modulates *spa* repression by suppressing transcription of *sarS*, an activator of *spa* expression in *S. aureus*. In contrast, the pathway by which *sarA* represses *spa* transcription is *sarS*-independent.

Contrary to the finding of Tegmark *et al* (63), we did not find that *sarS* represses *hla* transcription to a significant degree in a *sarA* mutant of strain RN6390. Whether other modulators of *hla* expression (*sarT*, *rot* and *sae*) are involved in this interaction is not clear and is the subject of current investigation.

3.3.5. The *sarU* and *sarY* loci

SarU and SarY are each 247-residues in length, with molecular sizes of 29.276 and 29.792 kDa, respectively. Both are basic proteins with respective pI of 9.81 and 9.34. Similar to SarS, SarU and SarY can each be considered as a molecule with two-halves each of which shares sequence similarity to the smaller homologs such as SarA, SarR and SarT (81). The significance of the sequence alignment is discussed below in the context of crystal structure. The *sarU* gene is adjacent to *sarT*. It is reasonable to speculate that these two genes may interact with each other. Although we do not know the exact function of SarY, it is likely that *sarY* may participate in some regulatory roles as well.

3.3.6. Other SarA homologs

Rot has sequence similarity to other SarA homologs. With the exception of the two-domain proteins (e.g. SarS), Rot, at 153 residues (18 kDa), is the largest. Contrary to other SarA homologs, the pI of Rot is acidic at 5.08. Conceivably, this may well reflect a divergent mode of regulatory mechanism. Besides Rot, other SarA homologs include SarV (116-residue, 13.9 kDa) and SarX (141-residue, 16.7 kDa). Based on our experience with

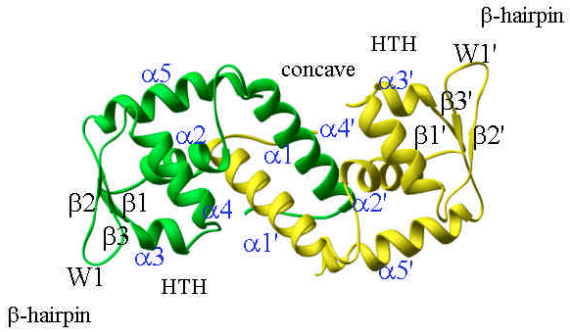

Figure 6 The crystal structure of SarR. One SarR monomer is colored Green while the other is Yellow. View of concave side along the dimer 2-fold axis of the SarR dimer, subdomain 1 (winged-helix motif) contains β1, α3, α4, β2, β3 from one monomer, subdomain 2 (winged-helix motif) contains β1, α3, α4, β2, β3 from the other, subdomain 3 (helical core) contains α1, α2, α5 from both monomers.

known SarA homologs, we predict these proteins to participate in the SarA/*agr* regulatory pathway. We also realize that, if the stringency of the search (E value ≤ 0.01) is further relaxed, additional hits from the *S. aureus* genome may emerge. To maintain proper focus, we will limit our discussion to these SarA homologs.

3.4. The crystal structures of SarR and SarA and their implications on the SarA protein family

To understand the structural basis for the binding and activation mechanism of target promoters by members of the SarA protein family, we recently solved the crystal structure of SarR (81). SarR is a dimeric structure comprising 5 α-helices, 3 β-strands and several loops (α1α2-β1α3α4-β2β3-α5) (81) (Figure 6). Extensive hydrophobic interactions occur between two α1 helices from each monomer. Additional hydrophobic interactions also take place between the N-terminus of one monomer and the C-terminus of the other to form a very tight dimer that can be only be disrupted with chaotropic agents (e.g. 4 M urea). There is a short turn between α3 and α4 that constitutes a typical helix-turn-helix motif (HTH). Deletion analysis indicated the HTH motif is essential to SarA function (73). In addition, this motif is also homologous to the DNA binding domain of VirF (72), a well-described transcriptional regulator of virulence factors in *Shigella flexneri* (82). In the middle of the SarR dimer is a canyon-like structure. Many Lys and Arg residues, although randomly distributed throughout the SarR molecule, are located on this side to form a positively charged track in the dimeric structure (81). Interestingly, many of the basic residues (Lys52 and Lys61, Lys80, Arg82, and Arg88 of SarR), predicted to be involved in the DNA binding on the concave side (HTH and β-hairpin loop), are conserved among many members of the SarA family (Figure 7). In contrast, residues involved in the activation domain on the convex side (e.g. Asp6, Asp9, Glu108, Glu110 and Glu111) are less conserved, consistent with the notion that the SarA protein family may activate diverse gene targets. The spatial arrangement of the two HTH modules in the SarR dimer is similar to the catabolite activator protein (CAP), a member of the winged-helix family (83). In this modeling scheme, the three β-strands and the HTH motif in each monomer form the typical winged-helix motif while the central helices (α1α2α5α1'α2'α5') comprise the core, which acts as a scaffold to hold the winged-helix motifs. Modeling of SarR to DNA, based upon the CAP protein, predicts that the HTH makes contact to the major groove of DNA while the β-hairpin loop binds to the minor groove. This mode of binding by SarR differs from the typical wing-helix family in which the β-hairpin loop is too short to bind to the minor groove (84). Using the CAP structure as a model, the longest direct distance for the concave side (DNA binding side) of the SarR dimer is ~71Δ, corresponding to 2 DNA turns (20 nt). This model also predicts the dimeric surface to hold a stretch of bent DNA ~27 bp in length, which translates to ~92Δ for normal B-form DNA. This is in agreement with the footprinting data, showing that SarR binds to a 29-bp sequence in the target promoter (85). The predicted bend in target promoter DNA, upon SarR binding, may reflect a regulatory mechanism for the SarA protein family to control gene transcription.

Schumacher *et al.* recently described the crystal structures of SarA and a SarA-*agr* DNA complex (86). Like SarR, SarA exists as a dimer, with each of the monomers consisting of four α-helices, and two loose regions comprising a β-hairpin turn and a C-terminal loop (α1α2α3-β1β2 plus the C-loop). Hydrophobic interactions between two monomers occur primarily via two α1 helices while the β-hairpin and C-loop are loosely tethered to the helical core. Despite SarA being a DNA binding protein (24, 77), no obvious DNA binding pockets or motifs are evident. In contrast to SarR, residues assigned to DNA binding (residues 92-96, 102-103, 110 and 113-114) are not conserved in the SarA protein family. Despite a wider binding site, the SarA-DNA co-crystal was determined with a 7-bp duplex overhang, showing an unaltered core while marked conformational changes in the β-hairpin and C-loop are predicted, resulting in a rigid structure involving encasement of target DNA by the α4 helix. This model predicts the over-winding of bound DNA in the minor groove and hence is consistent with the genetic data that spatial shortening between –10 and –35 in the target *agr* P3 promoter from 20 to 17-bp converts repression to activation (87).

Surprisingly, despite significant homology and conservation of residues involved in DNA binding between SarA and SarR, the two reported structures differ significantly. Notably, most residues in the inducible region (residues 71-75,94-95 and 103-114) of SarA are not absolutely conserved in the SarA family. By contrast, residues 86-88 and important basic residues within the HTH motifs of SarR, implicated in DNA binding, are conserved among the SarA family members. Although the binding of SarA to a 7-bp duplex was found to be accompanied by marked conformational changes (86), we have failed to observe SarA binding to this 7-bp *agr* promoter sequence in gel shift assays. Because the DNA

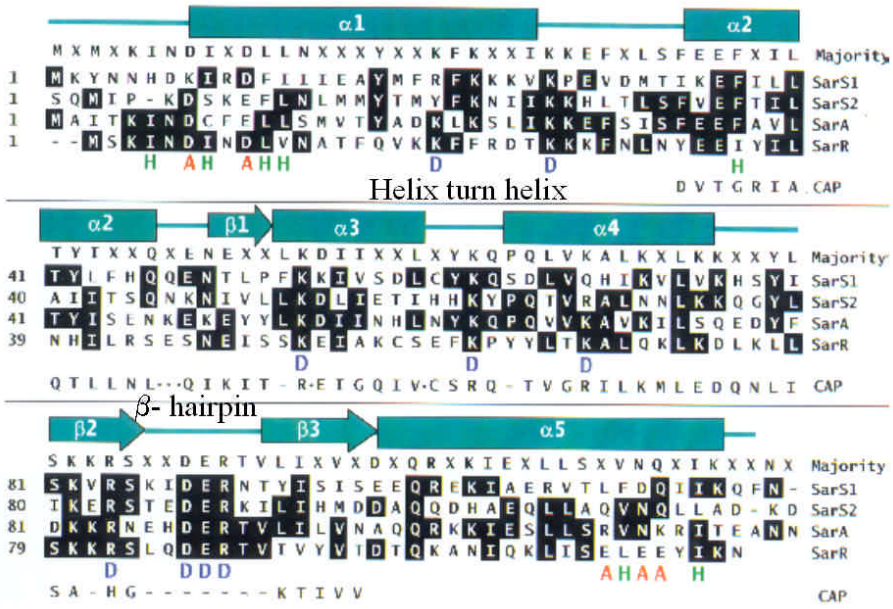

Figure 7 Superimposing the secondary structure of SarR upon the sequence alignment of SarA and three SarA homologs. Numbers at the beginning of each line indicate amino acid positions relative to the start of each protein sequence. Helices are indicated by rectangles, β-sheets or loops are indicated by lines. "H" represents residues that take part in dimerization. "A" indicates residues that may be part of the activation motifs. "D" implicates residues that may be involved in the interactions of SarR with DNA. The sequence of DNA-binding motif from CAP is also aligned to the SarA homologs. Dot means residue or residues omitted, dash means residue or residues missed in CAP. SarS2 starts at 1 (actual position on SarS is 126).

fragment used in the SarA-DNA co-crystal is so short and many of the SarA residues proposed to be involved in DNA binding are not conserved in the SarA family, the applicability of this model to molecules in solution would require additional biochemical confirmation data. As we also lack the SarR-DNA co-crystal structure, we anticipate future studies on SarR-DNA crystal structure, coupled with targeted mutagenesis, will allow us to derive a general picture of promoter activation/repression by the SarA family of regulatory proteins.

3.5. Environmental and host factors in global regulation of virulence determinants:

3.5.1. The role of environmental factors in global regulation

Environmental factors play a role in *sarA* and *agr* expression. For instance, 1 M NaCl has been shown to disrupt RNAIII transcription (75, 88-90). Chan and colleagues have also delineated an *agr* independent pathway for *spa*, *hla* and *tst* repression in the presence of 1 M NaCl (88). Likewise, glucose appears to down-modulate *agr*-related genes, possibly as a result of catabolite repression of the *agr* locus (91). Glycerol monolaurate, a surfactant, has been found to inhibit postexponential phase activation of virulence factors (e.g. exotoxins), without affecting *agr* expression (92). In the absence of any exogenous supplements in minimal medium, the pH of a *S. aureus* culture generally decreases as the cells transition from exponential to postexponential phase; however, if the pH of the culture is kept at 5.5, RNAIII expression will decrease further than cultures maintained at pH 6.5 (91). Thus, besides growth phase signals, environmental factors such as oxygen, nutrients, salt, osmolarity and pH may provide sensing signals for the bacteria in changing growth environments. Whether proteins in the SarA family play an intermediary role in response to these environmental stimuli are not well defined. Importantly, many of these studies utilized strain 8325-4 (16, 88, 93, 94), an isolate with a known defect in one of the anti-Sigma factor genes (*rsbU*) within the *sigB* operon (14, 95). As most of these studies were conducted in batch cultures with many independent variables (e.g. growth phase, pH, glucose limitation and altered oxygen tension, diffusion of antibiotics in above culture conditions), the interpretation of these data based upon a single environmental variable should be made with caution. Finally, these *in vitro* studies also did not take into account the host forces at play *in vivo* (e.g. host proteins, phagocytic cells and local microenvironment within specific tissues).

3.5.2. The role of host factors in global regulation

To correlate *in vitro* findings with those *in vivo*, our laboratory has constructed a shuttle plasmid containing the promoterless green fluorescent protein reporter gene (gfp_{uv}), a reporter system useful for both *in vitro* and *in vivo* analyses (50, 96). Using this strategy, various *sarA* promoter fragments was inserted upstream of the *gfp* reporter gene in a shuttle plasmid followed by electroporation into *S. aureus* strain RN6390 (96). Activation of these colonies *in vitro* disclosed that only the *sarA* P1 and the combined P2-P3-P1 promoters display green fluorescence while the *sarA* P2 and the ΦB-

dependent P3 promoters yield very little fluorescence (96). We next introduced intravenously *S. aureus* bacteria containing the *sarA* P1, P2 or P3 GFPuv fusions in a rabbit endocarditis model to assess *in vivo* gene activation (96). The results revealed that the *sarA* P1 promoter, at 24 h after infection, is active *in vitro* and *in vivo*. In contrast, the P3 promoter is inactive in both instances. Surprisingly, the P2 promoter is silent *in vitro* but becomes highly active *in vivo*. Besides different patterns of promoter activation, the same promoter activated in one tissue may not be activated in another. For instance, the *sarA* P2 promoter was activated in heart valve tissues but remained silent in the kidney in this model despite the prevalence of bacteria in both tissues (96). By contrast to cardiac vegetations, the kidney, as an area of high pH and osmolarity, likely represents a microenvironment not conducive to *sarA* P2 activation. The activation of a particular promoter also depends on the location within a single target tissue. In particular, the *sarA* P2 promoter was found to be active only on the surface on the cardiac vegetation where the cells are metabolically active but not in the depth of the vegetation where the bacterial cells are metabolically quiescent (96). These comparative data emphasize the differential activation of *sarA* promoters between *in vivo* and *in vitro* settings.

Georke *et al.* recently monitored *agr* RNAIII transcription of bacteria harvested from the sputum of cystic fibrosis (CF) patients (96a). Quantitation of the RNAIII transcript was performed by competitive RT-PCR. Surprisingly, RNAIII was poorly expressed, indicating a relatively inactive *agr* operon in the CF sputum *in vivo*. Despite a low level of RNAIII expression, the transcription of *hla*, a target gene of *agr*, was observed in many sputum samples, thus suggesting that other regulatory circuits aside from *agr* may be operative *in vivo* (96a). Alternatively, the local bacterial densities within CF sputums may not be high enough for quorum sensing to occur. More recently, Yarwood *et al.* utilized DNA microarray to evaluate the expression of *agr* in serum and in implanted subcutaneous chambers in rabbits (96b). As compared to expression in culture medium, RNAIII transcription was dramatically repressed in serum and *in vivo*, despite increased expression of secreted virulence factors sufficient to cause toxic shock syndrome in the animals. Contrary to the invasive syndromes of *S. aureus*, TSS is a non-invasive disease that is toxin mediated. Therefore, the direct application of this finding to invasive staphylococcal diseases is not clear.

Based on these *in vivo* expression studies, it is evident that the bacteria respond to selective host microenvironments often in context of a gene subset rather than as a specific virulence determinant. It is also our contention that many of these global regulators, including members of the SarA protein family, may serve as conduits to host signals for the bacteria. The bacteria, in turn, will respond by turning on a selective subset of genes to maximize survival within hosts.

3.6. The role of global regulators in virulence

As many of the virulence factors (e.g. hemolysins and fibronectin binding proteins) are regulated by *sarA* and *agr* (97), the contributory roles of these two regulators to virulence have been evaluated in several animal model systems including the rabbit endocarditis model (97, 98), the endophthalmitis model (99-101), the murine arthritis model (102, 103), the rabbit osteomyelitis model (104), the murine subcutaneous infection model (42) and, more recently, the murine brain abscess model (105). The results of these published studies will not be described in full detail here. However, it suffices to say that all of these animal studies serve to demonstrate that *sarA* and *agr* mutants and, in particular, the double *sarA*/*agr* mutant, are less able to cause infections than the parental control. Nevertheless, it is of interest that the adherence of the double *sarA*/*agr* mutant (30 min after infection) to the heart valve in the rabbit endocarditis model, which represents an acute/subacute endovascular infection model, is reduced (97). These animal studies support the notion that *sarA* and *agr* are important regulatory loci in the control of virulence gene expression in *S. aureus* including those involved in initial adherence (e.g. *fnb* controlled by *sarA*) (97) and intravegetation persistence and multiplication (e.g. *hla* by *sarA* and *agr*) in the fibrin-platelet matrix on the valvular endothelium. With so many virulence determinants altered in these mutants, these studies clearly emphasize the multifactorial nature of pathogenicity in *S. aureus* (15).

Contrary to *sarA* and *agr*, *sigB* has not been found to play major roles in acute models of infections (106). It has been argued that activation of SigB expression in acute infections, with its down-modulating effect on *hla*, *fnb* and *coa* expression (via reduced SarA) (15), will be detrimental to the "well being" of the bacterium. However, the role of SigB in persistent chronic infections (e.g. chronic abscess, osteomyelitis) has not been clearly defined since these infections are more indolent and likely occur without a dramatic outburst of hemolysins and enzymes, virulence factors essential to acute microbial propagation. Finally, small colony variants of *S. aureus* have been observed in patients with chronic and persistent *S. aureus* infections (e.g. cystic fibrosis lung, chronic but low-grade bacteremia and osteomyelitis) (107). Some of these small colony variants have been linked to menadione, hemin and thymidine auxotrophies (108, 109). Whether the small colony phenotype is modulated by regulatory loci such as *sigB*, *agr* and the SarA protein family has not been fully determined.

3.7. The role of *sarA* and *agr* in apoptosis

Chronic *S. aureus* infections are frequent in patients with diabetes (110) and cystic fibrosis (108, 111). It has been proposed that internalization of *S. aureus* by host cells may mediate persistent infections since the intracellular milieu, with its protected microenvironment, may shield the bacteria against antibiotics and antimicrobial agents (112, 113). More specifically, recent studies have shown that *S. aureus* can be internalized by bovine mammary epithelial cells (113), chicken osteoblasts (114), human endothelial cells (112) and bronchial epithelial cells from a Cystic Fibrosis patient (50) (115). Apoptosis of these cells would then ensue. Using GFP as a reporter, we recently showed that *S. aureus* is not a passive

bystander in the internalization process, but rather actively replicates in a bronchial cystic fibrosis cell line (50). In contrast to necrosis, apoptosis is an innate cell suicide mechanism that proceeds without significant inflammatory responses (116). Pathogen-induced apoptosis may serve to evade host defenses that may otherwise limit the extent of bacterial infections. Studies by Wesson *et al.* revealed that *agr* and *sarA* mutants were internalized by cultured bovine mammary epithelial cells at levels greater than the parental strain but, unlike the parent control, failed to induce apoptosis (113). Thus, *sarA* and *agr* regulated proteins are critical to the induction of apoptosis in bovine mammary epithelial cells. Because of tissue and host cell specificity, it is not clear whether similar *S. aureus*-induced apoptotic pathways regulated by *sarA* and *agr* would apply to other cell lines such as human endothelial cells and epithelial cells, especially those lining the normal respiratory epithelia.

4. PERSPECTIVES

The *sarA* and *agr* loci are an integral part of a regulatory circuitry that coordinately controls the expression of virulence determinants in *S. aureus*. The multifactorial nature of *S. aureus* pathogenicity implies that it may be useful to target a pleiotropic regulator rather than a specific determinant for the development of novel antimicrobial agents. Based on interactions with *agr*, the *sarA* regulatory system can be divided into three distinct pathways for target gene activation: 1) the *hla* pathway (via *agr*); 2) the *spa* pathway (co-repression with *agr*); and 3) the *fnb* pathway (*agr*-independent). In identifying a conserved SarA binding sequence among *sarA* target genes such as *agr*, *hla*, *spa* and *fnbA*, we propose a unifying hypothesis whereby SarA, the major *sarA* regulatory molecule, binds to the conserved sequence upstream of the -35 promoter box of target genes to activate (e.g. *agr*) or repress (e.g. *spa*) gene transcription.

In scanning the *S. aureus* genome, we found at least 9 other SarA homologs, which we called the SarA protein family. With the exception of Rot, SarS, SarU and SarY, many of these homologs are small (153 residues or less) basic proteins. Rot is a small acidic protein while SarS, SarU and SarY can be envisioned as protein structures with two-halves each of which shares sequence similarity to the smaller SarA homologs. Structural studies of SarR and SarA indicate that many of the SarA homologs likely exist as dimers. The SarR dimer is a three-domain structure with a central helical core and two winged-helix motifs, structural features that are commonly found among eukaryotic members of the winged-helix family of regulatory proteins (84, 117). Within each winged helix are a HTH motif and a β-hairpin loop, both predicted to be DNA binding domains (81, 84). However, contrary to the mammalian counterparts where the HTH motifs bind the minor groove and the β-hairpin loop interacts with the major groove, the vice-versa is true for SarR (84). Because of this finding, we hypothesize that the SarA family may represent a unique subclass of the winged-helix family. As the basic residues within the HTH and the β-hairpin loop are highly conserved within the SarA protein family, we speculate that family members may bind to target promoter DNA in a similar fashion. On the other hand, a lack of conserved acidic residues within the putative activation domain also implies that members of this protein family likely activate target genes differentially, possibly reflecting adaptation of the bacteria to diverse growth environments.

Superimposed upon these regulatory elements are host signals that may perturb SarA and subsequently *agr* expression, presumably via *in vitro*-associated regulators (a few described above) or via novel control pathway that can only be detected with *in vivo* selection system. As the host milieu, with an interplay of tissue factors and host defense forces, is complex, an exciting part of future staphylococcal research clearly lies in novel approaches that identify virulence genes expressed primarily *in vivo*.

The coordinate expression of these host-induced genes in *S. aureus* is likely to be precise since the sequence with which specific genes are expressed is critical to microbial survival. In this context, it is highly appropriate to envision bacterial response as a subset of genes activated by the host microenvironment. Clearly, SarA and *agr* are regulatory elements that control the expression of virulence determinants *in vivo* as demonstrated in several animal studies (42, 98-105). The discovery of additional SarA homologs in *S. aureus* has significantly increased the complexity of the regulatory circuit. Our challenge will be to ascertain how these regulators interact with one another, in particular within the context of host milieu. Once the regulatory cascade is ascertained, it will be exciting to determine the specific environmental and host signals to which these regulators respond. Understanding these regulatory mechanism will place us at the exciting threshold of deciphering the precise interplay of microbial and host factors. With the impending threat of vancomycin resistance in *S. aureus*, understanding the inner workings of these regulatory genes may provide the theoretical basis for designing new antimicrobial agents and preventive vaccines.

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