

BACKGROUND AND SIGNIFICANCE

***Staphylococcus aureus* as a human pathogen.** *S. aureus* is an opportunistic pathogen capable of causing diverse infections ranging from superficial and relatively benign infections of the skin to serious and even life-threatening disease (41). The most serious are the deep-seated infections that arise either after invasion of the bloodstream from primary sites of infection or after the direct introduction of *S. aureus* as a result of trauma. Specific examples include osteomyelitis and endocarditis, both of which involve the colonization of a solid-surface substratum (41). These infections are extremely difficult to resolve for two reasons. **The first** is the continued emergence of *S. aureus* strains that are resistant to multiple antibiotics (34). Indeed, in an increasing number of cases, the only treatment option is the glycopeptide antibiotic vancomycin. Moreover, reports describing the isolation of *S. aureus* strains that are relatively resistant to vancomycin emphasize the tenuous nature of our reliance on this antibiotic (K. Hiramatsu, 1997 Gordon Conference on Staphylococci and Staphylococcal Diseases, Andover, N.H.). **The second** complicating factor is the formation of a bacterial biofilm on the solid-surface substratum (Fig. 1). Because the biofilm is an effective impediment to antibiotic delivery, resolution of deep-seated *S. aureus* infections typically requires surgical intervention to debride the infected tissue and/or remove the offending implant.

We believe our proposal has relevance with respect to the development of new therapeutic agents and with respect to the delivery of those agents to the site of infection. Specifically, we believe that *sar* may be an appropriate target for the development of antimicrobial agents capable of attenuating the virulence of *S. aureus* and that these agents may, by virtue of their ability to interfere with the coordinated regulation of *S. aureus* virulence factors (see below), inhibit biofilm formation and thereby increase the efficacy of conventional antimicrobial agents. **Moreover, recent evidence suggests that therapeutic strategies directed at *sar* may have a direct impact on the resistance of *S. aureus* to at least some antimicrobial agents.** For instance, Bayer et al. (2) suggested that transcription from the *sar* P₃ promoter may be dependent on the *S. aureus* stress-response sigma factor σ^B .

This section describes the importance of the research to public health. It is written in less technical terms that can be understood by all reviewers. Bolding highlights key concepts and allows reviewers to scan the pages and retrieve information quickly during the review meeting.

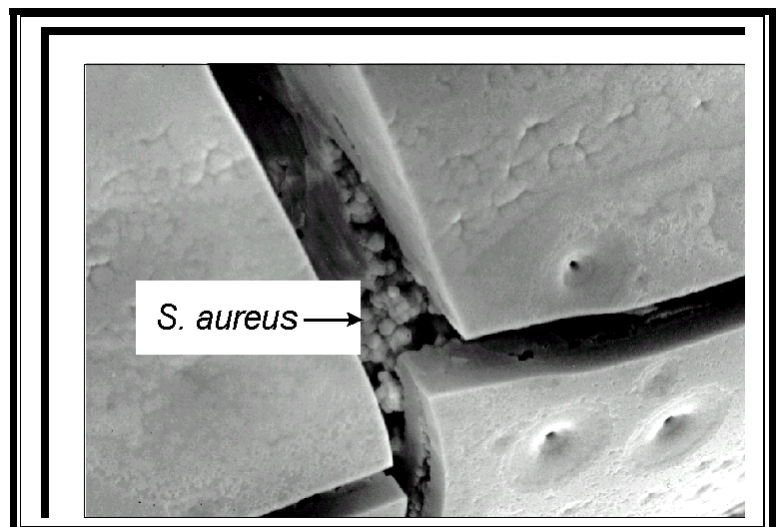


Fig. 1. *S. aureus* growing within a biofilm. The electron micrograph was prepared from the bone of an experimentally-infected rabbit (18).

A figure illustrates a point in the description.

That is a significant observation since Wu et al. (43) demonstrated that inactivation of the *sigB* operon in the homogenously-resistant *S. aureus* strain COL results in a 64-fold increase in the susceptibility to methicillin (i.e. a 64-fold decrease in the methicillin MIC). The observation that *sar* mutants exhibit a small but reproducible increase in the susceptibility to methicillin (44) supports the hypothesis that the inability to express *sar* may contribute to the decline in methicillin resistance.

The extensive background shows a broad understanding of the field, its gaps, opportunities, and the significance of the proposed research.

Phenotypic switching in the pathogenesis of *S. aureus*. The pathogenic potential of *S. aureus* is due to its capacity to produce a diverse array of virulence factors in a coordinately-regulated fashion. These factors can be broadly divided into two groups based on whether they remain associated with the cell surface or are exported into the extracellular milieu. This distinction is significant because the two groups are globally and inversely regulated, with expression of the genes encoding surface proteins (e.g. coagulase, protein A) occurring under conditions that do not warrant expression of the genes encoding extracellular virulence factors. *In vitro*, this differential regulation is manifested as the expression of surface proteins during exponential growth and the expression of exoproteins during the post-exponential growth phase (Fig. 2). The post-exponential phase shift to exoprotein synthesis is associated with a coordinately-regulated decrease in the synthesis of surface proteins and is thought to have an *in vivo* corollary that roughly translates to before and after formation of an abscess (Fig. 2).

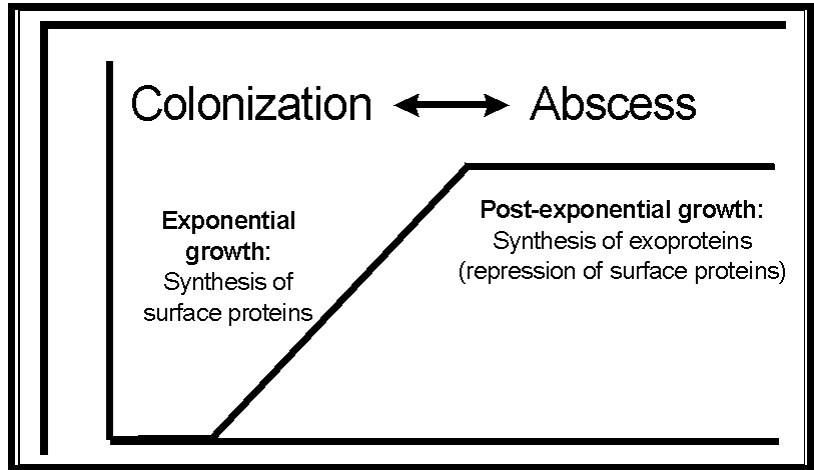


Fig. 2. Schematic representation of *S. aureus* phenotypic switching *in vitro* and its potential relevance to growth *in vivo*.

Specifically, it has been hypothesized that *S. aureus* surface proteins are expressed during the early stages of infection when the most important considerations for the bacterium are avoiding recognition by host defenses and colonizing an appropriate target tissue (36). In contrast, the production of extracellular toxins and degradative enzymes is most important when the cell density becomes high enough to result in a localized immune response, limited nutrient availability and a reduced growth rate. Presumably, the cell density is sufficiently high only within an abscess or biofilm. It has also been suggested that exoproteins may promote the eventual escape from an abscess, at which point the phenotype reverts to surface protein expression as the bacterium attempts to colonize a new site (36). **The significance of the reversible switch between expression of surface proteins and expression of exoproteins is evidenced by the fact that *S. aureus* mutants unable to regulate this phenotypic switch consistently exhibit reduced virulence in animal models of staphylococcal disease (3, 8, 10, 20, 24, 28, 40).**

A figure summarizes a point in the text.

Regulatory elements controlling expression of *S. aureus* virulence factors.

To date, there are reports describing five different exoprotein-deficient, *S. aureus* mutants (9, 12, 31, 33, 40). All five were originally defined by chromosomal transposon insertions. With the exceptions of the staphylococcal accessory regulator (*sar*) and the accessory gene regulator (*agr*), these mutants have not been characterized beyond localization of the transposon insertion and a phenotypic description. These phenotypic reports must be interpreted with caution. For example, the transposon insertion in the extracellular protein regulator (*xpr*) was reported to result in a phenotype identical to *agr* mutants (40). Because the *xpr* mutant produced reduced amounts of the *agr*-encoded RNAII and RNAIII transcripts (22), it was concluded that the regulatory effect of the *xpr* mutation was probably a function of its impact on *agr*. However, it was not possible to complement the *xpr* mutation even after the introduction of an extensive set of overlapping clones derived from the corresponding chromosomal region of the parent strain (M. Smeltzer, unpublished observation). Ji et al. (38) subsequently described the isolation of exoprotein-deficient *S. aureus* strains arising from spontaneous mutations within *agr*. In fact, there is evidence to suggest that *agr* contains mutational hotspots (42). These reports prompted a re-examination of the *xpr* mutant and the subsequent discovery that the exoprotein-deficient phenotype was due to a previously undetected frameshift mutation within *agrC* rather than any defect associated with the transposon insertion (J. Landolo, personal communication). A similar explanation may account for the phenotype observed with other transpositional mutants. However, the *sar* and *agr* loci have been cloned and sequenced, and there is an extensive body of data conclusively establishing that these loci function as primary mediators of the regulatory events controlling expression of *S. aureus* virulence factors (8,10,11,18,28). **We believe that *sar* may play a particularly important role in that it can modulate the production of *S. aureus* virulence factors both by modulating the activity of *agr* and by direct interactions with specific target genes.** The remainder of this section is devoted to a description of the *sar* and *agr* loci and the experimental data supporting that hypothesis.

Note the good use of references, including preliminary, unpublished data, which are also listed in Literature Cited section. Bolding main points allows reviewers to scan the pages and retrieve information quickly during the review meeting.

The background and significance leads to the rationale for the hypothesis, which is presented again.

The *staphylococcal accessory regulator (sar)*. The *sar* locus spans 1349 bp and encodes three overlapping transcripts (*sarA*, *sarB* and *sarC*), all of which include the entire *sarA* coding region (2). Expression of each transcript is growth-phase dependent, with expression of *sarA* and *sarB* being highest during exponential growth and expression of *sarC* being highest during

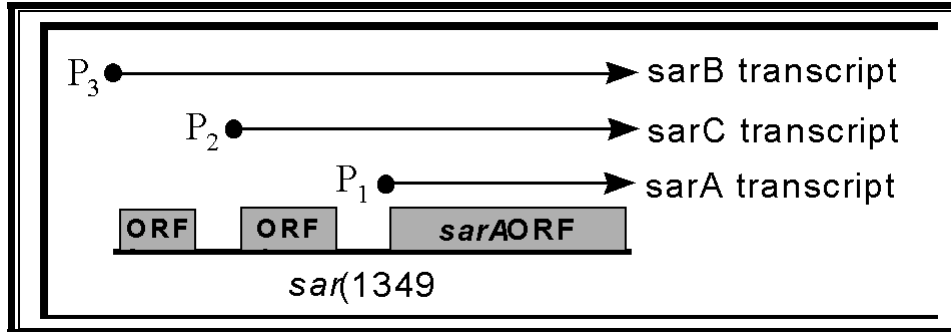


Fig. 3. Schematic representation of the *sar* locus showing the relative location and size of each transcript. The P₃, P₂ and P₁ promoters (filled circles) were defined by Bayer et al. (2). Shaded boxes indicate open-reading frames (ORFs). ORF₃ and ORF₄ potentially encode peptides with 18 and 39 amino acids respectively. The *sarA* ORF in RN6390 encodes a protein with 339 amino acids. The production of each *sar* transcript is growth-phase dependent as described in the text.

the post-exponential growth phase (2, 7). Although the only recognized protein product of the *sar* locus is SarA (2), the *sarB* and *sarC* transcripts encode short open-reading frames that are not present in the *sarA* transcript (Fig. 3). Moreover, there is evidence to suggest that the different *sar* transcripts serve different functional roles. For instance, *sar* mutants produce reduced amounts of alpha-toxin and increased amounts of lipase (11). Heinrichs et al. (23) demonstrated that introduction of the region encoding the *sarA* transcript results in complementation of the alpha-toxin deficiency while restoration of lipase production to wild-type levels is dependent on introduction of the region encoding the longer *sarB* transcript (23). The *sarB* transcript also appears to be more efficient than the *sarA* transcript with respect to augmenting transcription from *agr* P₂ and P₃ promoters (see below). These functional differences could arise from the differential production of SarA from each transcript coupled with variations in the amount of SarA required to exert a regulatory effect on different target genes. Alternatively, it is possible that the short ORFs contained within the *sarB* and *sarC* transcripts encode peptides that somehow modulate the activity of SarA (2, 13). We will address the first of these possibilities by correlating the production of each *sar* transcript with the accumulation of SarA (Specific Aim #1, Part A). We will address the second possibility by correlating the accumulation of SarA with the activity of SarA as a DNA-binding protein (Specific Aim #1, Part B) and as a transcriptional activator (Specific Aim #1, Part C).

Figure is essential to understanding the transcripts, regulation, and the proposed research.

The accessory gene regulator (*agr*). The focus of this proposal is on the *sar* regulatory locus with a particular emphasis on SarA. However, an important reason for that focus is the recent observation that **SarA functions as a transcriptional activator of the genes encoded within *agr* by virtue of its ability to bind *cis* elements upstream of the *agr* P₂ and P₃ promoters (27).** The following discussion is intended to emphasize the significance of that observation.

The *agr* regulatory system consists of two genetically and functionally-linked loci (16). One of these (the *agrBDCA* operon; hereinafter referred to as the *agr* operon) encodes a two-component signal transduction system (Fig. 4). The two-component system is a “quorum-sensing” system that is induced when the cell density of *S. aureus* populations reaches a threshold level (38). The *agr* operon is transcribed as a polycistronic mRNA

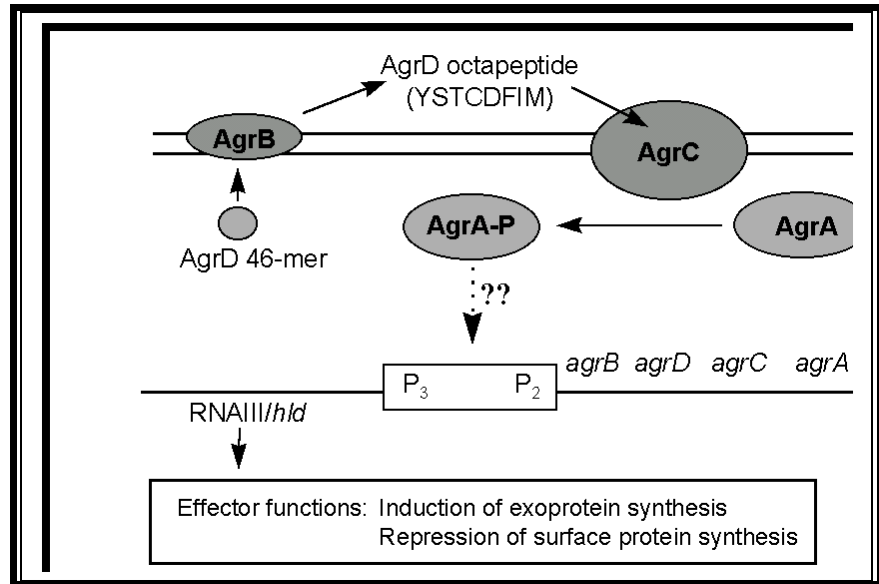


Fig. 4. Schematic representation of the *agr* regulatory system. The *agrBDCA* operon encodes a “quorum-sensing” system (see text) that modulates production of the RNAIII effector molecule. The function of each component and the uncertainty associated with the interaction between phosphorylated AgrA and the P₂/P₃ promoter region are discussed in the text.

(RNAII) from a promoter designated P₂. The *agrA* and *agrC* genes encode the response regulator and the sensor protein of the two-component system respectively (Fig. 4). The *agrD* gene encodes a 46 amino acid peptide that is processed by AgrB to an octapeptide pheromone during its passage across the cell membrane (38). Once the cell density reaches a sufficient level, the extracellular concentration of the pheromone becomes high enough to activate the membrane-embedded AgrC sensor resulting in phosphorylation of the intracellular AgrA response regulator. Phosphorylation of AgrA results in the autocatalytic induction of RNAII synthesis and induces transcription from a second, divergently-transcribed promoter (P₃) located ~120 bp upstream of P₂ (27). The P₃ promoter controls expression of a regulatory RNA designated RNAIII (Fig. 4). The RNAIII transcript includes the *S. aureus* alpha-toxin gene (*hld*), however, it is the RNAIII transcript itself, rather than any protein encoded within the *agr* operon or within RNAIII, that functions as the effector molecule of the *agr* regulatory system (16, 29, 36). RNAIII serves a dual regulatory role in that its production is associated with repressed synthesis of surface proteins (e.g. coagulase, protein A) and enhanced synthesis of extracellular toxins and enzymes (35). In most cases, the mechanism by which RNAIII exerts its regulatory effect is unknown (see below). Nevertheless, the observation that *S. aureus* mutants unable to produce RNAIII are consistently less virulent than their wild-type parent strains (3, 8, 10, 20, 24, 28, 40) clearly establishes the significance of RNAIII in the pathogenesis of staphylococcal disease. **The relevance of that observation to this proposal arises from the fact that mutation of *sar* results in reduced RNAIII synthesis (23).**

Figure provides a model for the proposed studies.

The interaction between SarA and agr. The scenario described above suggests that AgrA is a DNA-binding protein that activates transcription from the *agr* P₂ and P₃ promoters. However, attempts to demonstrate that AgrA binds *cis* elements upstream of the *agr* P₂ or P₃ promoters have been unsuccessful (37). These results suggest that AgrA is necessary, but not sufficient, for the transcriptional activation of RNAIII synthesis. That suggestion prompted the search for an accessory protein that binds the intergenic region between the *agr* P₂ and P₃ promoters. The first indication that *sar* might encode such a protein came from Heinrichs et al. (23), who demonstrated that 1) *sar* mutants produce reduced amounts of RNAII and RNAIII, 2) the production of RNAII and RNAIII is restored when an intact *sarA* gene is introduced into a *sar* mutant and 3) cell extracts from wild-type strains contain a protein that binds the *agr* P₂ promoter region while extracts from *sar* mutants do not. These results were extended by Morfeldt et al. (27), who demonstrated that cell lysates from wild-type strains contained a protein that binds *cis* elements upstream of both *agr* promoters and that the bound protein has an N-terminal sequence consistent with SarA. Morfeldt et al. (27) also demonstrated that **SarA binding to *cis* elements upstream of the *agr* P₃ promoter is required for the induction of RNAIII synthesis and the regulated expression of the *agr* target genes encoding alpha-**

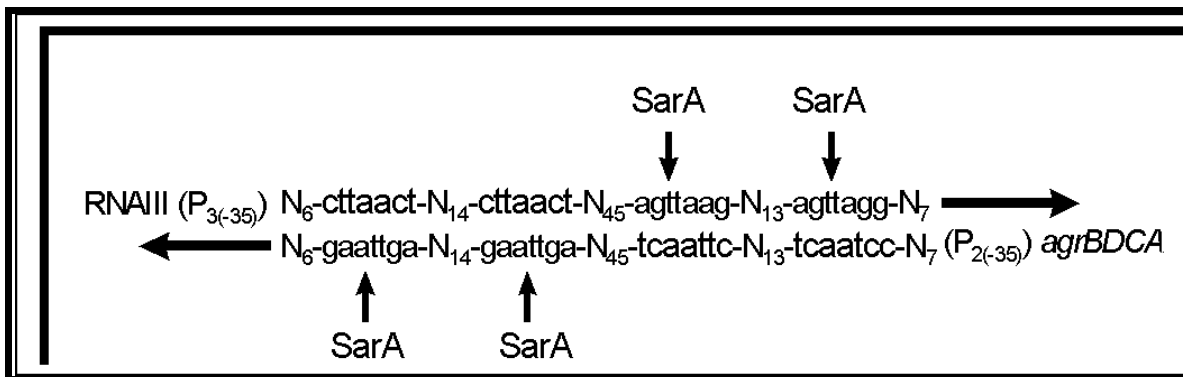


Fig. 5. Heptanucleotide repeats within the intergenic region between the *agr* P₂ and P₃ promoters. All heptanucleotide sites are indicated as potential SarA-binding sites although the stoichiometry of SarA binding is unknown.

toxin (*hla*) and protein A (*spa*) (27). Electrophoretic mobility shift assays (EMSA) suggested that SarA binds to a 7 bp site (AGTTAAG) that occurs as a perfect repeat upstream of the *agr* P₃ promoter and as an imperfect repeat (AGTTAGG) upstream of the *agr* P₂ promoter (27). In both cases, the first of the 7 bp sites is centered 31 bp upstream of the -35 site while the second is located 13-14 bp downstream of the first (Fig. 5). The observation that the repeats upstream of the P₂ promoter have an orientation opposite to those upstream of the P₃ promoter is consistent with the observation that RNAII and RNAIII are divergently transcribed. Although the mechanism remains unclear, Morfeldt et al. (27) suggested that SarA binding may bend the DNA in a fashion that facilitates an undefined interaction with AgrA and the subsequent induction of RNAIII transcription.

Figure provides a model for the proposed studies.

***sar* as an *agr*-independent regulatory element.** The data discussed above suggests that a primary function of SarA is to optimize RNAIII transcription. However, phenotypic comparison of *sar* and *agr* mutants indicates that SarA regulates expression of some *S. aureus* virulence factors in an *agr*-independent manner. For instance, while *agr* mutants exhibit an enhanced capacity to bind fibronectin and produce reduced amounts of lipase, *sar* mutants have the opposite phenotype (8). Moreover, the fact that *sar/agr* double mutants have a phenotype like that observed in *sar* mutants (8) suggests that the regulatory effect of *sar* on these target genes is epistatic to *agr*. Additionally, Cheung et al. (7) demonstrated that *sar* encodes a factor that represses expression of the protein A gene (*spa*) even in an *agr*-negative genetic background. Finally, our studies with the *S. aureus* collagen adhesin gene (*cna*) provide direct evidence for the existence of an *agr*-independent *sar* regulatory pathway. Specifically, we have established that transcription of *cna* is growth-phase dependent, with expression being highest during the exponential growth phase and falling dramatically as cultures enter post-exponential growth (20). Mutation of *agr* has little effect on the temporal pattern of *cna* transcription while mutation of *sar* results in a dramatic increase in *cna* transcription and a corresponding increase in the ability to bind collagen (20, 21). **Most importantly, we have demonstrated that the regulatory impact of *sar* on *cna* transcription involves a direct interaction between SarA and DNA targets upstream of the *cna* coding region (see Preliminary Results, Fig. 12).** These results are the first demonstration that SarA binds to DNA targets other than those associated with *agr*. Defining the SarA-binding site upstream of *cna* and the mechanism by which SarA binding represses *cna* transcription are a primary focus of this proposal (Specific Aim #2).

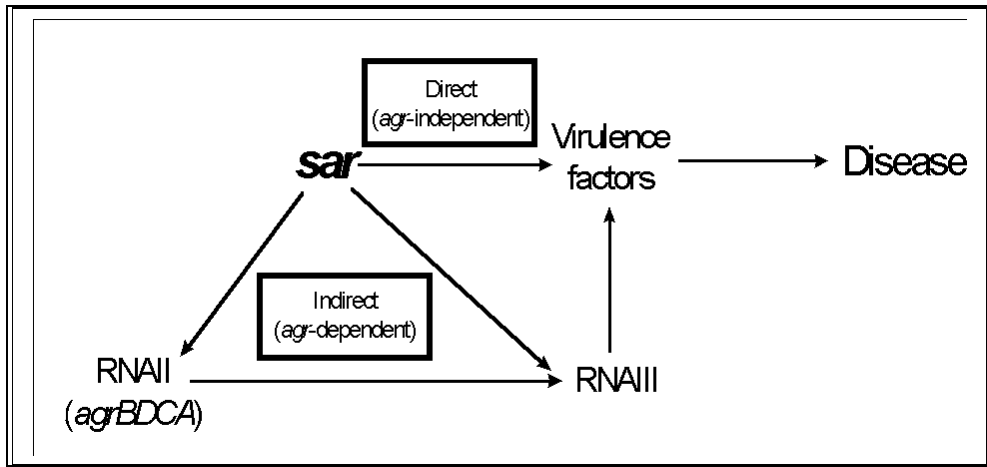
Note how investigator's previous studies are incorporated into this section illustrating expertise in this area.

The contribution of sar to the pathogenesis of S. aureus.

Based on the recognized impact of SarA on *agr* transcription (27) and our results indicating that SarA modulates *cna* transcription through a direct interaction with *cis* elements upstream of *cna*, we conclude that the SarA-mediated regulation of *S. aureus* virulence factors involves both *agr*-dependent and *agr*-independent pathways. The *agr*-dependent pathway may provide *S. aureus* with an independently-regulated mechanism of inducing the production of RNAIII. Such a system would allow for the production of exoproteins even when the cell density is too low to induce RNAIII production via the accumulation of the AgrD pheromone. The *agr*-independent pathway may provide *S. aureus* with a mechanism by which it can fine tune its phenotype to take maximum advantage of the growth conditions encountered within the host. The importance of the *agr*-independent pathway is evident in the results of animal studies assessing the virulence of *sar* mutants. For example, mutation of *sar* results in reduced virulence in animal models of

staphylococcal disease (8, 10, 28). However, because mutation of *agr* also results in reduced virulence (8, 18, 24, 40), it is possible to explain these results based on the regulatory impact of SarA on transcription of the genes encoded within *agr*. **On the other hand, the impact of SarA on *agr* transcription does not explain the observation that, in at least two animal models of staphylococcal disease (8, 24), *sar/agr* double mutants were shown to have reduced virulence even by comparison to *agr* mutants.** Such a synergistic reduction in virulence is consistent with a scenario in which *sar* functions through both *agr*-dependent and *agr*-independent regulatory pathways (Fig. 6). The experiments described in this proposal will allow us to assess both the nature and the scope of the *agr*-independent pathway by 1) defining the parameters required for the production of functional SarA (Specific Aim #1), 2) elucidating the mechanism by which *sar* regulates the transcription of *cna* (Specific Aim #2) and 3) identifying additional *S. aureus* genes under the direct regulatory control of SarA (Specific Aim #3).

Alternative interpretations lead to additional research questions.



Graphic provides quick summary and illustrates model.

Fig. 6. Summary of the impact of *sar* on expression of *S. aureus* virulence factors. The *agr*-dependent pathway is designated “indirect” because the effect on expression of the target gene is mediated through an intermediary, presumably RNAIII. The *agr*-independent pathway is designated “direct” because it is independent of the regulatory impact of SarA on *agr*.

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