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SPECIFIC AIMS

Staphylococcus aureus is a well-armed opportunistic pathogen that produces a diverse array of virulence factors and causes a correspondingly diverse array of infections. The pathogenesis of *S. aureus* infections depends on the coordinately-regulated expression of two groups of virulence factors, one of which (surface proteins) allows the bacterium to evade phagocytes and colonize host tissues while the other (extracellular toxins and enzymes) promotes survival and multiplication at a localized site of infection. Our long term goal is to elucidate the regulatory mechanisms controlling expression of these virulence factors as a prerequisite to the development of therapeutic protocols that can be used to attenuate the disease process. **The specific hypothesis behind the proposed research is that the staphylococcal accessory regulator *(sar)* is a major regulatory switch controlling expression of *S. aureus* virulence factors.** That hypothesis is based on the following observations. **First**, *sar* encodes a DNA-binding protein (SarA) required for expression of the *agr*-encoded RNAIII regulatory molecule (27). The SarA-dependency of RNAIII expression is important because RNAIII modulates expression of many *S. aureus* virulence factors (29). **Second**, phenotypic comparison of *sar* and *agr* mutants indicates that *sar* also regulates expression of certain *S. aureus* genes in an *agr*-independent manner (11, 21). An example of particular relevance to this proposal is the *S. aureus* collagen adhesin gene (*cna*). **Third**, mutation of *sar* results in reduced virulence in animal models of staphylococcal disease (8, 10, 28). Moreover, as anticipated based on the preceding discussion, *sar/agr* double mutants have reduced virulence even by comparison to *agr* mutants (8, 24). **Based on these observations, the experimental focus of this proposal is on the *sar* regulatory locus.** The specific aims are designed to provide a comprehensive assessment of the *agr*-independent regulatory functions of *sar*:

1. Correlate the production of each sar transcript with the production of functional SarA. The only recognized protein product of the *sar* locus is the SarA DNA-binding protein. However, Northern blot analysis reveals three *sar* transcripts (sarA, sarB and sarC), all of which include the entire *sarA* gene. Expression of each transcript is growth-phase dependent. The functional significance of this differential regulation will be assessed by correlating the production of each transcript with the production and activity of SarA.

A. The temporal production of SarA will be assessed by Western blot of *S. aureus* whole cell extracts with an affinity-purified anti-SarA antibody.

Uses short sentences, is neat, is clean with no typographical errors. Uses bullets and numbered lists for effective organization. No header or footer, since those are added automatically later. Stays within page limit **(in original version, prior to annotation.)**

Specific aims start with the background for the informed non-expert, writing at about the level of *Scientific American*. Gives summary for non-primary reviewers. Puts less technical information first.

Hypothesis is easy to locate in bold type and includes health importance of the project. Provides three reasons for hypothesis, with references.

Scope of research is limited to three specific aims listed in bold and followed by a brief description of how each aim will be accomplished. These aims are the steps designed to prove the hypothesis.

B. The DNA-binding activity of SarA will be assessed by electrophoretic mobility shift assays (EMSA) using whole cell extracts and DNA fragments known to include SarA-binding sites (e.g. *cis* elements upstream of the *agr* P_2 and P_3 promoters).
C. The function of SarA as a transcriptional activator will be assessed *in vivo* using transcriptional fusions between each of the *agr* promoters and a promoter-less *xylE* reporter gene.

Lists give guideposts to reviewers; indents and bold add readability.

2. Characterize the mechanism of sar-mediated regulation of the S. aureus collagen adhesin gene (cna). We have established that *sar* is the primary regulatory element controlling *cna* transcription and that this effect involves a direct interaction between SarA and *cis* elements upstream of *cna*. However, unlike SarA binding to the *agr* promoter region, SarA binding represses *cna* transcription. We will correlate the production of each *sar* transcript with the production of SarA and with the regulation of *cna* transcription. We will also define the *cis* elements upstream of *cna* that constitute the SarA DNA-binding target.

A. Complementation of the *cna* transcriptional defect will be done by introducing plasmids encoding the sarA, sarB or sarC transcripts into a *cna*-positive *sar* mutant. Once the SarA-binding site upstream of *cna* has been defined (see below), the complementation studies will be correlated with SarA binding to *cis* elements upstream of *cna*.
B. The SarA DNA-binding site(s) upstream of *cna* will be localized by EMSA using purified SarA. The specific binding site(s) will be identified by DNA footprinting and characterized by EMSA using *cna* sequence variants and purified SarA.
C. The *in vivo* significance of SarA binding will be assessed using transcriptional fusions between sequence variants of the *cis* elements upstream of *cna* and a promoter-less *xylE* reporter gene.

3. Identifify S. aureus virulence factor genes under the direct control of SarA. The scope of SarA as a regulatory protein is not well-defined because the identification of SarA targets has been restricted by the availability of gene probes and/or appropriate phenotypic assays. Our successful purification of SarA in a form capable of binding appropriate DNA targets (e.g. *cis* elements upstream of *agr* and *cna*) will allow us to define the DNA determinants required for SarA binding using a functional selection. We will then identify SarA binding sites within the *S. aureus* genome and evaluate SarA regulation of the genes *cis* to these binding sites.

A. PCR-assisted binding site selection will be used to functionally select DNAs with SarA binding sites from a random pool of synthetic DNA fragments. The consensus binding site will be determined by computer-assisted alignment of functionally selected DNAs.
B. The consensus sequence for a SarA-binding site will be used in homology searches of existing *S. aureus* genomic databases. The search will be extended to include the entire *S. aureus* genome as it becomes available.
C. SarA regulatory control of the genes *cis* to putative SarA-binding sites will be tested by Northern blot analysis of wild-type strains and their corresponding *sar* mutants.

S. aureus is among the most persistent of all human pathogens. The continued emergence of antibiotic-resistant strains emphasizes the need to identify new therapeutic targets for the treatment of *S. aureus* infections. We believe the *sar* regulatory locus may be an appropriate target in that disruption of *sar*-mediated regulation has the potential to attenuate the bacterium to the point that it is more susceptible to clearance either by the normal host defense systems or existing antimicrobial agents. Accomplishing the specific aims outlined in this proposal will provide the foundation required to assess that possibility by establishing the correlation between *sar* transcription and SarA production and activity (Specific Aim #1), elucidating the mechanism

by which *sar* controls expression of a specific target gene (*cna*) (Specific Aim #2) and identifying additional SarA targets within the *S. aureus* genome (Specific Aim #3).

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