Stephen P. Heyse, M.D. **SUMMARY STATEMENT** *Release Date:* 12/01/2004
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Application Number: 1 R01 AI43356-01

Review Group: BM-2
BACTERIOLOGY & MYCOLOGY SS SUBCOM 2
Meeting Date: FEB/MARCH 1999
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Project Title: *sar*-mediated regulation in *Staphylococcus aureus*
SRG Action: *Priority Score:* 130 *Percentile:* 3.0
Human Subjects: 10-NO HUMAN SUBJECTS INVOLVED
Animal Subjects: 10-NO LIVE VERTEBRATE ANIMALS INVOLVED
GENDER, MINORITY, & CLINICAL TRIAL CODES NOT ASSIGNED

PROJECT YEAR	DIRECT COSTS REQUESTED	ESTIMATED TOTAL COST
01	175,000	175,000
02	175,000	175,000
03	175,000	175,000
04	175,000	175,000
05	175,000	175,000
TOTAL	875,000	875,000

ADMINISTRATIVE BUDGET NOTE: The budget shown is the requested budget and has not been adjusted to reflect any recommendations made by reviewers. If an award is planned, the costs will be calculated by Institute grants management staff based on the recommendations outlined below in the COMMITTEE BUDGET RECOMMENDATIONS section.

RESUME AND SUMMARY OF DISCUSSION: This is an outstanding proposal to investigate the contribution of specific regulatory loci to global virulence factor gene expression in an important pathogen, Staphylococcus aureus. The investigator has presented a well founded hypothesis based on important prior studies of both the sar and agr loci, new preliminary data, has developed reagents that are critical for performing the experimental studies proposed in each aim and is therefore poised to potentially develop important information relevant to virulence factor gene expression.

DESCRIPTION (adopted from the application): Staphylococcus aureus is an opportunistic pathogen that produces a diverse array of virulence factors and causes a correspondingly diverse

array of infections. Our long-term goal is to elucidate the regulatory mechanisms controlling expression of these virulence factors as a necessary prerequisite to the development of therapeutic protocols capable of attenuating the disease process. The specific hypothesis is that the staphylococcal accessory regulator (sar) is a major regulatory locus controlling expression of S. aureus virulence factors. We base that hypothesis on the observations that 1) sar encodes a DNA-binding protein (SarA) required for expression of the agr-encoded, RNAIII regulatory molecule,

2) phenotypic comparison of sar and agr mutants demonstrates that sar also regulates expression of S. aureus virulence factors in an agr-independent manner and 3) mutation of sar and agr results in reduced virulence even by comparison to agr mutants. Based on these observations, the experimental focus of this proposal is on the agr-independent branch of the sar regulatory pathway. The specific aims are to: 1. Define the relationship between sar transcription and the production of functional SarA. We will correlate the production of the SarA, SarB, and SarC transcripts with (i) the production of SarA, (ii) the DNA-binding activity of SarA and (iii) the ability of SarA to regulate transcription of a target gene. 2. Characterize the mechanisms of sar-mediated regulation of cna transcription. The S aureus collagen adhesin gene (cna) is expressed in a growth-phase dependent manner and that sar is the primary regulatory element controlling can transcription. Preliminary experiments indicate that the regulatory impact of sar on cna transcription involves a direct interaction between SarA and DNA upstream of cna. We will identify the sar transcripts required to complement the cna defect and will correlate the results of our complementation studies with the production and
activity of SarA. We will also identify and characterize the cis elements that define cna as a target for sar-mediated regulation. 3. Identify S. aureus genes under the direct regulatory control of SarA. We will (i) characterize the consensus SarA-binding site, (ii) identify putative SarA targets within the S. aureus genome and (iii) confirm the sar-mediated regulation of these targets by Northern blot analysis of sar mutants.

CRITIQUE 1:

SIGNIFICANCE: This is a very well written proposal by an investigator with experience investigating the molecular basis of virulence factor expression by Staphylococcus aureus. The primary hypothesis of this research focuses on the role of the staphylococcal accessory regulator (sar) as a major regulatory switch controlling expression of Staphylococcus aureus virulence factors. The investigator has provided an excellent summary of the background and significance of this work, pointing out the current issues regarding the
relationship of the different regulatory genes, i.e. agr and sar, on
the expression of exoproteins and cell surface proteins involved
in staphylococcal
virulence.

This proposal will further develop our understanding of the
regulatory switching mechanisms utilized by staphylococci under
various growth conditions relevant to the in vivo environment. Specifically, it will investigate the interaction of two major regulatory loci, agr and sar at the transcriptional level investigating the molecular interaction of sar on agr promoters. This is an extremely
important facet of pathogenesis research towards developing an
understanding of how environmental stimuli change the expression of
the plethora of virulence factors elaborated by this organism. Another
arm of this
proposal will investigate the control of sar as a regulatory element on a
collagen binding protein gene thought to be relevant in the
pathogenesis of infections as osteomyelitis. Sar functions independently in the control of the cna gene from its interaction with the agr locus.

APPROACH: After providing an excellent description as to what is known about the interaction of the sar and agr loci, including excellent schematics to clarify the complex interaction of these two regulatory elements, the investigator provided a well articulated series of preliminary results in support of the specific aims. This includes: cloning and expression of the SarA gene product demonstrating their ability to purify the protein to homogeneity, generation of monospecific SARA antiserum, construction of a reporter fusion vector critical for evaluating the affect of sar on specific promoters cloned in front of the xylE reporter gene, and finally, additional studies on the role of sar in controlling expression of the collagen adhesin gene independent of the agr regulatory locus. Isogeneic mutants for sar and agr expression in combination or independently have also been derived as reagents for investigating the role of the sar regulatory element.

In regards to the regulation of cna, the investigator and co investigators have demonstrated that the cna gene is found only is specific isolates of Staphylococcus aureus. Although this gene is not found on a transposable element it appears to insert in the chromosome in a specific region downstream of a 500 base pair region, which appears to be important in regulation of its expression. This regulatory locus is not upstream of an interrupted open reading frame and is also found in S. aureus strains that lack the cna gene. This upstream region will be the focus of one of the specific aims of this proposal.

Some concern is raised in the data shown in figure 10. The investigator states that agr has less of a regulatory influence on cna expression than sar, however, the agr mutation in the strain 6911 appears to result in higher derepressed cna (collagen binding) expression, observed for either the U-175 mutant (sar-) or the U-172 mutant (agr-/sar-) mutant. This raises some concern as to whether there may be some strain variability in the role that agr may play in cna expression versus sar regulation. Despite this discrepancy the data does support the notion that sar may have more of a role in controlling cna expression, as demonstrated by Northern blot analysis, at least with the isogeneic mutant strains.

Lastly, preliminary data is provided on cloning the DNA regions encoding the sar locus which includes the A, B, and C transcripts. This is a complex operon as it appears that SarB and SarC are often co-transcribed with SarA, because they are 5 of the SarA gene. The role of the B and C open reading frames on the potential activity of SarA and the genes it regulates are another focus of this proposal.

In specific aim 1, the investigator proposes to define the relationship between sar transcription and the production of SarA. The overall rationale is to correlate temporal production of the A, B and C transcripts, along with activity of the SarA gene product. The experimental design utilizing Western blot and EMSA analysis to define expression of sar transcripts and SarA gene products in various mutant backgrounds, are well defined and well controlled. It is important that the strain RM6911 is included in these studies not only for the reasons stated by the investigator concerning the role of the agr locus, but also because it shows that mutation of the agr locus may actually have an important role in cna gene expression. Determining the kinetics of sar accumulation by Western blot during different growth phases is important and the experiments are well described. The investigator may want to consider whether growth phase is the only environmental condition which may influence SarA expression. For example, growth rate has also been shown to have a profound influence on expression of some virulence factors in streptococci. The DNA binding studies will be correlated to the temporal accumulation of SarA using electrophoretic mobility shift assays. The investigator states that these studies offer the possibility of defining whether the short SarB and SarC transcripts may be important for SarA function. However, no experiments specific to defining the

contribution of the B and C orfs independent of A are described. The overall approach to the EMSA studies, however, is well thought out and conceived.

Lastly, the role of the SarA gene product, as a DNA binding protein involved in transcriptional activation, will be investigated using the reporter fusion vector derived in the preliminary data. These are excellent studies and should pose no problems and will provide important information as to the interaction of the sar protein with the promoters of the agr regulatory locus.

In aim 2, the investigator will begin to investigate and characterize the mechanism by which sar regulates expression of the S. aureus collagen adhesin gene. This regulation is thought to be independent of agr and will focus on the 5 region to the cna gene, thought to be present in all strains of Staphylococcus aureus independent of the presence of the cna gene. Well described experimentation to evaluate complementation of the defect in cna to transcription observed in sar mutants and characterization of the SarA DNA binding sites upstream of cna in an attempt to correlate SarA binding with regulation of cna transcription are provided. One concern in these studies is that the complementation experiments using the cloned DNA fragments from the sar loci will consist of SarA open reading frame or the SarA reading frame with either SarB or SarC open reading frames. Unfortunately, plans to potentially clone the SarB or SarC orfs independently for evaluation in the complementation experiments is not provided. Independent experiments with each individual orf could provide interesting results as to the contribution of each of these open reading frames to SarA activity. This may be important in characterizing the DNA binding sites upstream of the cna gene as these open reading frames could potentially influence binding to promoters that are independent of those found in the agr loci.

In the experiments correlating SarA binding with regulation of cna transcription these studies will utilize the cna targets cloned 5 of the xylE reporter gene vector. Reporter assays will then be utilized under various stages of growth with either the wild type binding sites or mutant binding sites. The investigator states that this will be done in a cna negative, S. aureus strain such as RN6390, to serve as a negative background for upstream background binding. However, as described earlier in the proposal, the 500 base pair region upstream of the cna integration site contains these same endogenous binding sites in cna negative strains. How will this obviate the background concerns the investigator raises? Does RN6390 lack the 500 base pair binding site region of interest?

Lastly, in aim 3 the investigator proposes to identify other S. aureus virulence factors which may be under the direct control of SarA. Using a PCR based-assisted selection binding site strategy, the investigator plans to identify other DNA binding regions which interact with SarA from the genome of S. aureus in the hopes of identifying other genes which may be under the coordinate regulation of SarA. This may provide a clue to other virulence genes not only important in pathogenesis, but under direct control of this regulatory element. The methods are elegantly described and the inclusion of Dr. Hurlburt strengthens the likelihood of the success of this aim. This is an ambitious aim and the investigator may be advised to consider smaller scale pilot studies for a future proposal. Again, one of the concerns raised in this aim is the possibility that the SarB and SarC open reading frames may influence SarA binding, requiring more specific investigation of the SarB and SarC gene products independent of their expression with SarA.

INNOVATION: The proposed studies are elegantly designed and build upon the investigator s expertise. Although the methods proposed are not new, their application to a very important pathogen towards our understanding of the regulatory elements involved in virulence factor expression during the pathogenesis of disease is extremely important. The complexity and the possibility that regulatory elements may have coordinate and independent effects on virulence factor gene expression requires the type of complex

experimental investigation proposed in this application. The design is well focused and promises to yield important new information that may be generally applicable to other gram-positive pathogens.

INVESTIGATORS: Dr. Mark Smeltzer is the principal investigator and has experience in molecular biology including complementation studies with the sar and cna genes, and transcriptional analysis using reporter gene fusions. He has had excellent training in the laboratory of Dr. John Iandolo and has been productive in description of important virulence genes and their transcriptional regulation in S. aureus. Dr. Barry Hurlburt is a biochemist with significant experience characterizing DNA binding sites for repressor proteins and brings to this proposal a wealth of expertise important to the sar DNA binding site studies. Dr. Gillaspy has recently obtained her PhD under the investigator's supervision and will continue as a research associate defining the mechanisms by which sar regulates cna gene expression.

ENVIRONMENT: No concerns. Most of the reagents, strains and mutants are already available and both investigators are in close proximity inviting close collaborative interaction. No major equipment is required that is not already available within the department of the principal investigator and coinvestigator.

OVERALL EVALUATION: Some concern is raised over the experiments involving the SarB and SarC open reading frames and their contribution to the regulatory activity of SarA. In addition, the investigator may want to develop a background chromosomal mutation in the strain RN6390 removing the 500 bp region containing the putative cna regulatory binding sites found in all S. aureus strains to minimize background SAR binding problems. Specific aim 3 is very ambitious, but stands to identify virulence genes under the regulation of the sar locus, however, the investigator may need to postpone or attempt pilot studies as part of a future proposal. Overall, important new insights into the co-regulation and independent regulation of virulence factor gene expression in Staphylococcus aureus could result from this investigation.

CRITIQUE 2:

SIGNIFICANCE: S. aureus is a major pathogen which has suffered from a lack of insightful analysis of molecular pathogenesis. This proposal is a significant step toward unraveling how S. aureus adapts to human environments, such as in osteomyelitis. A critical virulence determinant has been identified, the collagen binding protein, and its relationship to the sar regulatory locus is solid. These tools are the first pair of regulator/ effector for S. aureus that can be cleanly analyzed in vivo and in vitro so as to yield other virulence effectors. Work from other labs has tried to get this far but failed.

APPROACH: This is an exceptionally well written and clearly organized proposal to study a transcription factor (SarA) controlling virulence genes in S. aureus (including the collagen binding protein).

Specific Aim 1: The SarA protein arises from a gene with three start site variants. This aim will determine the relative participation of these three variants in producing functional SarA. This set of experiments is directed toward the ability of SarA to act directly to modify gene transcription rather than the focus other leading Staph labs have taken which involves indirect effects of sar through agr. Three subaims will seek a correlation of SarA protein, SarA DNA binding activity and SarA transcriptional activation of target genes. This approach will cut through the confusion in the field generated by following every possible effect of SarA at once and thereby not understanding any. The investigator has all the necessary strains and constructs in hand and the experiments will yield important information.

Specific Aim 2: The investigator has identified the only gene yet known to be directly regulated by SarA, cna. This allows determination for the first time of the DNA sequence targeted by SarA using standard techniques of analysis (such as DNA footprinting) in the cna upstream region. A

correlation of DNA binding to rate of gene transcription will be confirmed using an existing fusion construct.

Specific aim 3 will extend the surefire experiments of Aims 1 and 2 toward a model of disease by seeking other genes targeted for regulation by SarA. This will be done by defining the SarA consensus binding sequence by allowing purified SarA to bind to random PCR sequences. The capability of generating this library (SELEX technique) is already in place in the Co-investigators lab for similar purposes. This sequence will then be used to search the Staph genome to identify any genes proceeded by the consensus and therefore potentially in the SarA regulon. Their true regulation will be confirmed by traditional molecular biology techniques.

INNOVATION: The investigator has a unique tool, a purified SarA that binds DNA. He also has insight into infection processes through his work with osteomyelitis and thus his investigations can remain focused on human disease rather than trascriptional regulation per se. Finally, the collaboration with the coinvestigator gives access to a novel SELEX library and technique which is proven effective in isolating the transcription factor binding domains sought in this proposal.

INVESTIGATOR: Dr. Smeltzer is a new Assistant Professor on the faculty of University of Arkansas. He has experience in investigation of Staph virulence determinants and is fully versed in Staph genetics following a highly successful postdoc in J. Iandolo's lab at Kansas. These skills are useful in the present study. His publication record is very strong and demonstrates that the experiments planned are well within the area of his expertise. He has existing funds to study Staph elements important in osteomyelitis and this grant complements those studies by focusing on the regulation of such virulence determinants.

ENVIRONMENT: Dr. Smeltzer has set up an excellent collaborative effort to examine the structure of Staph regulatory elements with Dr. Hurlburt at the same institution. Dr. Hurlburt is well versed in this type of investigation.

OVERALL EVALUATION: This is a very strong proposal which will make an important impact on understanding Staph aureus pathogenesis by identifying and characterizing a key virulence regulon. The tools are in place, the technology is familiar to the investigator and co-investigator and the experimental design is logical and solid. The focus of the program of virulence genes as opposed to regulatory mechanisms per se is a great strength for NIAID study sections.

BUDGET: Both time and amount are appropriate and are recommended as requested.
