Annotated R01 Grant Application – NIAID

Last updated on January 11, 2005.

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Introduction

One of the most difficult tools to find -- and one of our most requested -- is an example of a well-written NIH grant application.

We are truly indebted to Dr. Mark Smeltzer for permitting us to use his outstanding basic science application, which he wrote as a new investigator in 1998, to help the next generation of investigators write their applications. Please note that the application is copyrighted.

Dr. Smeltzer's application appears as he submitted it to NIH except for changes we made to some forms to reflect PHS 398 version 09/2004. For example, we changed the budget request to a modular budget.

Further, we've added annotations to explain how this application reflects much of the advice we give in our "All About Grants" Web tutorials.

All our advice is the opinion of NIAID staff scientists and should be taken as our advice only. Differing opinions may exist, including those of NIH peer reviewers.

For more information, visit the NIAID Funding Web site:
http://www.niaid.nih.gov/ncn/

Form Approved Through 09/30/2007 O MB No. 0925-0001

Department of Health and Human Services
Public Health Services

Grant Application

Do not exceed character length restrictions indicated.

LEAVE BLANK—FOR PHS USE ONLY.

Type	Activity	Number
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 81 characters, including spaces and punctuation.)*
sar-mediated regulation in Staphylococcus aureus

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
(If "Yes," state number and title)
Number: Title:

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR — **New Investigator** ☒ **No** ☐ **Yes**

3a. NAME (Last, first, middle)
Smeltzer, Mark Stephen

3b. DEGREE(S)
BS, MS, PhD

3h. eRA Commons User Name
mssmeltzer

3c. POSITION TITLE
Assistant Professor

3d. MAILING ADDRESS *(Street, city, state, zip code)*
Department of Microbiology and Immunology
University of Arkansas for Medical Sciences
4301 Markham, Slot 511
Little Rock, Arkansas 72205-7199

3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Microbiology and Immunology

3f. MAJOR SUBDIVISION
College of Medicine

3g. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: (501) 686-7958 FAX: (501) 686-5359

E-MAIL ADDRESS:
smeltzermarks@exchange.uams.edu

4. HUMAN SUBJECTS RESEARCH
No ☒ Yes ☐

4b. Human Subjects Assurance No.

4c. Clinical Trial ☒ No ☐ Yes

4d. NIH-defined Phase III Clinical Trial ☒ No ☐ Yes

4a. Research Exempt No ☐ Yes ☐

If "Yes," Exemption No.

5. VERTEBRATE ANIMALS ☒ No ☐ Yes

5a. If "Yes," IACUC approval Date

5b. Animal welfare assurance no.

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
07/01/98	06/30/03	$175,000	$257,000	$875,000	$1,286,000

9. APPLICANT ORGANIZATION
Name Mark S. Smeltzer
Address Dept. of Microbiology and Immunology
Univ. of Arkansas for Medical Sciences
4301 W. Markham, Slot 511
Little Rock, Arkansas 72205-7199

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☒ State ☐ Local
Private: → ☐ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
DUNS NO. 1716046242A1 Cong. District 02

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name Sheryl N. Goldberg, MBA
Title Director, Research Administration
Address Univ. of Arkansas for Medical Sciences
4301 W. Markham, Slot 636
Little Rock, Arkansas 72205-7199
Tel: (501) 686-5502 FAX: (501) 686-8359
E-Mail: goldbergsheryl@exchange.uams.edu

13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name Sheryl N. Goldberg, MBA
Title Director, Research Administration
Address Univ. of Arkansas for Medical Sciences
4301 W. Markham, Slot 636
Little Rock, Arkansas 72205-7199
Tel: (501) 686-5502 FAX: (501) 686-8359
E-Mail: goldbergsheryl@exchange.uams.edu

14. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PI/PD NAMED IN 3a. *(In ink. "Per" signature not acceptable.)* DATE

15. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)* DATE

DESCRIPTION: See instructions. State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project (i.e., relevance to the **mission of the agency**). Describe concisely the research design and methods for achieving these goals. Describe the rationale and techniques you will use to pursue these goals.

In addition, in two or three sentences, describe in plain, lay language the relevance of this research to **public** health. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

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 DNAbinding activity of SarA and (iii) the ability of SarA to regulate transcription of a target gene.
2. Characterize the mechanism 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 cna 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. Identifiy S. aureus genes under the direct regulatory control of SarA. We will (i) characterize consensus SarA-binding site, (ii) identify putative SarA targets within the S. aureus genome the sar-mediated regulation of these targets by Northern blot analysis of sar mutants.

PERFORMANCE SITE(S) (organization, city, state)

University of Arkansas for Medical Sciences, Little Rock, Arkansas

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	eRA Commons User Name	Organization	Role on Project
Smeltzer, Mark S.	mssmeltzer	University of Arkansas	Principal Investigator
Gillaspy, Allison F.		University of Arkansas	Research Associate
Hurlburt, Barry K.	bkhurlburt	University of Arkansas	Co-investigator
Lutz-Rechtin, Tammy K.		University of Arkansas	Research Associate

OTHER SIGNIFICANT CONTRIBUTORS

Name	Organization	Role on Project
None.		

Human Embryonic Stem Cells ☒ **No** ☐ **Yes**

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://stemcells.nih.gov/registry/index.asp. *Use continuation pages as needed.*

If a specific line cannot be referenced at this time, include a statement that one from the Registry will be used.

Cell Line

Disclosure Permission Statement. Applicable to SBIR/STTR Only. See SBIR/STTR instructions. ☐ Yes ☐ No

The name of the principal investigator/program director must be provided at the top of each printed page and each continuation page.

RESEARCH GRANT

TABLE OF CONTENTS

Appendix *(Five collated sets. No page numbering necessary for Appendix.)*

Check if Appendix is Included

Appendices NOT PERMITTED for Phase I SBIR/STTR unless specifically solicited. ☐

Number of publications and manuscripts accepted for publication *(not to exceed 10)* _____

Other items (list):

BUDGET JUSTIFICATION PAGE
MODULAR RESEARCH GRANT APPLICATION

	Initial Period	2nd	3rd	4th	5 th	Sum Total (For Entire Project Period)
DC less Consortium F&A	175,000 *(Item 7a, Face Page)*	175,000	175,000	175,000	175,000	875,000 *(Item 8a, Face Page)*
Consortium F&A	0	0	0	0	0	
Total Direct Costs	175,000	175,000	175,000	175,000	175,000	$ 875,000

Personnel

Mark S. Smeltzer (Principal Investigator, 30% effort) will be responsible for the overall design and implementation of the experiments including coordination of the experiments carried out in the PI'S and the Co-I's laboratories. The PI will be directly responsible for the bacteriological aspects of the project including the analysis of cell lysates, complementation studies with sar and cna and the transcriptional analysis of reporter gene fusions.

Barry K. Hurlburt (Co-Investigator, 20% effort) will oversee the experiments directed toward characterization of the SarA-binding sites upstream of cna and the implementation and analysis of the SELEX experiments.

Allison F. Gillaspy (Research Associate, 100% effort) will be directly responsible for most of the experiments described in this proposal including (i) correlation of SarA production and activity with the temporal pattern of cna transcription, (ii) functional analysis of the SarA-binding site upstream of cna and (iii) confirmation of the sar-mediated regulatory control of cna and additional targets within the S. aureus genome. 100% of her time will be devoted to this project.

Tammy L. Lutz-Rechtin (Research Associate, 75% effort) directed toward the successful completion of the experiments described in the proposal. She will place particular emphasis on the SELEX experiments.

Consortium

N/A

Fee (SBIR/STTR Only)

N/A

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
eRA COMMONS USER NAME	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY

NOTE: The Biographical Sketch may not exceed four pages. Items A and B (together) may not exceed two of the four-page limit. Follow the formats and instructions on the attached sample.

A. Positions and Honors. List in chronological order previous positions, concluding with your present position. List any honors. Include present membership on any Federal Government public advisory committee.

B. Selected peer-reviewed publications (in chronological order). Do not include publications submitted or in preparation.

C. Research Support. List selected ongoing or completed (during the last three years) research projects (federal and non-federal support). Begin with the projects that are most relevant to the research proposed in this application. Briefly indicate the overall goals of the projects and your role (e.g. PI, Co-Investigator, Consultant) in the research project. Do not list award amounts or percent effort in projects.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Mark S. Smeltzer	Assistant Professor
eRA COMMONS USER NAME mssmeltzer	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Washburn University, Topeka, KS	B.S.	1982	Biology
Kansas State University, Manhattan, KS	M.S.	1987	Microbiology
Kansas State University, Manhattan, KS	Ph.D.	1990	Microbiology
Kansas State University, Manhattan, KS	Post-doc	1990-1993	Microbiology

A. Positions and Honors.

Positions

- 1982-1984: Medical Technician, Department of Pathology, Kansas State University, Manhattan, KS.
- 1984-1987: Microbiologist I, Department of Pathology, Kansas State University, Manhattan, KS.
- 1987-1990: Graduate Research Assistant, Department of Pathology, Kansas State University, Manhattan, KS.
- 1990-1993: Postdoctoral Research Associate, Division of Biology and the Department of Pathology and Microbiology, Kansas State University, Manhattan, KS.
- 1993-present: Assistant Professor, Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, Arkansas.
- 1995-present: Section Head, Musculoskeletal Infection Group, University of Arkansas for Medical Sciences, Little Rock, Arkansas.

Honors

- Psi Chi National Honorary Society, Washburn University, Topeka, KS (1981)
- Ravin-Muriel Rogers Fellowship, Wind River Conference on Genetic Exchange (1990-1991) Kansas Health Foundation Postdoctoral Scholar, Kansas State University, (1991-1993) Phi Zeta Award for Excellence in Basic Research, Kansas State University, (1992) New Investigator Award, Orthopaedic Research Society (1995)
- Randall Award, Outstanding Young Faculty Member, South Central Branch of ASM (1996)

B. Selected peer-reviewed publications (in chronological order).

Rahaley, R.S., Dennis, S.M., and *Smeltzer, M.S.* 1983. Comparison of the enzyme-linked immunosorbent assay a complement fixation test for detecting *Brucella ovis* antibodies in sheep. *Veterinary Record,* 113:467-470.

***Smeltzer, M.S.,* Gill, S.R. and landolo, J.J.** 1992. Localization of a chromosomal mutation affecting expression of extracellular lipase in *Staphylococcus aureus. Journal of Bacteriology,* 174:4000-4006.

***Smeltzer, M.S.,* Hart, M.E., and landolo, J.J.** 1992. Quantitative spectrophotometric assay for lipase activity in *Staphylococcus aureus. Applied and Environmental Microbiology,* 58:2815-2819.

Hart, M.E., *Smeltzer, M.S.,* and landolo, J.J. 1993. The extracellular protein regulator *(xpr)* affects exoprotein and *agr* mRNA levels in *Staphylococcus aureus. Infection and Immunity,* 175:7895-7879.

***Smeltzer, M.S.,* Hart, M.E., and landolo, J.J.** 1993. Phenotypic characterization of *xpr,* a global regulator of extracellular virulence factors in *Staphylococcus aureus. Infection and Immunity,* 61:919-925.

***Smeltzer, M.S.,* Hart, M.E., and landolo, J.J.** 1994. The effect of lysogeny on the genomic organization of *Staphylococcus aureus. Gene,* 138:51-57.

Chapes, S.K., Beharka, A.A., Hart, M.E., *Smeltzer, M.S.,* and landolo, J.J. 1994. Differential RNA regulation by Staphylococcal enterotoxins A and B in murine macrophages. *Journal of Leukocyte Biology,* **55:533-529.**

Gillaspy, A.F., Hickmon, S.G., Skinner, R.A., Thomas, J.R., Nelson, C.L. and *Smeltzer, M.S.* 1995. Role of the accessory gene regulator *(agr)* in the pathogenesis of staphylococcal osteomyelitis. *Infection and Immunity,* 63:3373-3380.

***Smeltzer, M.S.,* Pratt, F.L., Jr., Gillaspy, A.F. and Young, L.A.** 1996. Genomic fingerprinting for the epidemiological differentiation of *Staphylococcus aureus* clinical isolates. *Journal of Clinical Microbiology,* 34:1364-1372.

***Smeltzer, M.S.,* Thomas, J.R., Hickmon, S.G., Skinner, R.A., Nelson, C.L., Griffith, D., Parr, T.R., Jr. and Evans, R.P.** 1997. Characterization of a rabbit model of staphylococcal osteomyelitis. *Journal of Orthopaedic Research,* **15:414-421.**

Gillaspy, A.F., Patti, J.M., Pratt, F.L., Jr., and *Smeltzer, M.S.* 1997. Transcriptional regulation of the *Staphylococcus aureus* collagen adhesin gene *(cna). Infection and Immunity,* 65:1536-1540.

***Smeltzer, M.S.,* Gillaspy, A.F., Pratt, F.L., Jr., and Thames, M.D.** 1997. Comparative evaluation of *cna, fnbA, fnbB* and *hlb* genomic fingerprinting for the epidemiological typing of *Staphylococcus aureus. Journal of Clinical Microbiology,* 35:2444-2449.

***Smeltzer, M.S.,* Gillaspy, A.F., Pratt, F.L., and landolo, J.J.** 1997. Prevalence and chromosomal map location of *Staphylococcus aureus* adhesin genes. *Gene,* 196:249-259.

Gillaspy, A.F., Patti, J.M., Pratt, F.L., Jr., and *Smeltzer, M.S.* 1997. The *Staphylococcus aureus* collagen adhesin-encoding gene *(cna) is* within a discrete genetic element. *Gene,* **196:239-248.**

Gillaspy, A.F., Lee. C.Y., Sau, S., Cheung, A.L., and *Smeltzer, M.S.* Factors affecting the collagen binding capacity of *Staphylococcus aureus. Infection and Immunity, submitted.*

C. Research Support.

Completed Research Support

Grant #96-046 07/01/1996 – 06/30/1998
Orthopaedic Research and Education Foundation
Global regulatory elements of *Staphylococcus aureus as* therapeutic targets for the treatment of osteomyelitis.

Major goal: To determine whether mutation of *S. aureus* regulatory loci attenuate the bacterium to the point that it is more susceptible to antibiotic clearance.
Role: PI

Grant #A1 37729 (R29) 07/01/1996 – 06/30/2001
National Institute of Allergy and Infectious Disease.
Role of adhesins in staphylococcal osteomyelitis.
Major goal: To define the *S. aureus* adhesins that promote the colonization of bone and the colonization of orthopaedic implants.
Role: PI

Pending Research Support

None.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Allison F. Gillaspy	Research Associate
eRA COMMONS USER NAME	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Hendrix College, Conway, AR	B.A.	1993	Biology
University of Arkansas for Medical Sciences	Ph.D.	1997	Microbiology

A. Positions and Honors.

Positions

- 1993-1997: Graduate Research Assistant, UAMS Department of Microbiology and Immunology, Little Rock, AR
- 1997-present: Post-doctoral Research Associate, UAMS Dept. of Microbiology and Immunology, Little Rock, AR

Honors

- Student Travel Award, Wind River Conference on Prokaryotic Biology, 1995, 1997
- ASM Student Travel Grant, Annual Meeting of the American Society for Microbiology, New Orleans, LA, 1996 College of Medicine Student Research Grant, 1996

B. Selected peer-reviewed publications (in chronological order).

***Gillaspy, A.F.,* Hickmon, S.G., Skinner, R.A., Thomas, J.R., Nelson, C.L. and Smeltzer, M.S.** 1995. Role of the accessory gene regulator *(agr)* in the pathogenesis of staphylococcal osteomyelitis. *Infection and Immunity.* 63:3373-3380.

Smeltzer, M.S., Pratt, F.L., Jr., *Gillaspy, A.F.,* and Young, L.A. 1996. Genomic fingerprinting for epidemiological differentiation of *Staphylococcus aureus* clinical isolates. *Journal of Clinical Microbiology.* 34:1364-1372.

***Gillaspy, A.F.,* Patti, J.M. and M.S. Smeltzer.** 1997. Transcriptional regulation of the *Staphylococcus aureus* collagen adhesin gene *(cna). Infection and Immunity.* 65:1536-1540.

***Gillaspy, A.F.,* Patti, J.M., Pratt, F.L., Jr., landolo, J.J. and Smeltzer, M.S.** 1997. The *Staphylococcus aureus* collagen adhesin encoding gene *(cna) is* within a discrete genetic element. *Gene.* **196:239-248.**

Smeltzer, M.S., *Gillaspy, A.F.,* Pratt, F.L., Jr., Thames, M.D. and landolo, J.J. 1997. Prevalence and chromosomal map location of *Staphylococcus aureus* adhesin genes. *Gene.* 196:249-259.

Smeltzer, M.S., *Gillaspy, A.F.,* Pratt, F.L., Jr. and Thames, M.D. 1997. Comparative evaluation of *cna, fnbA, fnbB* and *hlb* genomic fingerprinting for the epidemiological typing of *Staphylococcus aureus. Journal of Clinical Microbiology.* 35:2444-2449.

***Gillaspy, A.F.,* Sau, S., Lee, C.Y. and Smeltzer, M.S.** Factors affecting collagen binding capacity in *Staphylococcus aureus*. Manuscript submitted.

C. Research Support.

Completed Research Support

None.

Pending Research Support

None.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Barry K. Hurlburt	Associate Professor
eRA COMMONS USER NAME bkhurlburt	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Virginia	B.A.	1975-1979	Biochemistry
University of Virginia	Ph.D.	1987	Biochem/Genetics
Stanford University	Post-doc	1987-1990	Biochem/Genetics

A. Positions and Honors.

Positions

- 1977-1979: Independent researcher in the laboratory of Dr. Thomas H. Cromartie, Department of Chemistry, University of Virginia. Research: synthesis/characterization of suicide enzyme inhibitors.
- 1979: Advanced techniques in X-ray crystallography research course in laboratory of Dr. Eckard Sinn, Department of Chemistry, University of Virginia. Research: crystal structure studies of inorganic copper compounds.
- 1979-1981: Research Assistant to Professor Irwin R. Konigsberg, Department of Biology, University of Virginia. Research: protein differences between normal and dystrophic muscle cells.
- 1981-1986: Doctoral research in laboratory of Professor Reginald H. Garrett, Department of Biology, University of Virginia. Research: genetics and biochemistry of nitrate assimilation in *N. crassa.*
- 1987-1990: Postdoctoral research in laboratory of Professor Charles Yanofsky, Department of Biological Sciences, Stanford University. Research: structure/function analysis of *trp* repressor of E. *coli.*
- 1990-1996: Assistant Professor of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences.
- 1993-present: Program Member, Arkansas Cancer Research Center, Little Rock, AR.
- 1997-present: Associate Professor of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences. Research: Structure and function of proteins, particularly transcription factors.

Honors

- **Fellowships and Awards**
  - 1987-1990: NIH National Research Service Award
  - 1984-1986: NIH Predoctoral Traineeship in Regulatory Genetics
  - 1983-1984: Governor's Fellowship in Biology
  - 1982-1983: Governor's Fellowship in Biology
- **Professional Societies**
  - American Society for the Advancement of Science American Society for Microbiology
  - American Society for Biochemistry and Molecular Biology

B. Selected peer-reviewed publications (in chronological order).

Rodig, O.R., Bruekner, T., *Hurlburt, B.K.,* Schlatzer, R.R., Venable, T.L. and E. Sinn (1981) Relation between structure and spectra of pseudo-tetrahedral copper(II) complexes. Crystal structure of bis(2,2'-pyridyloamido) copper(II). *J.C.S. Dalton Transactions* 196-200.

***Hurlburt, B.K.,* and R.H. Garrett** (1988) Nitrate assimilation in *Neurospora crassa:* Enzymatic and immunoblot analysis of wild type and *nit* mutant protein products in nitrate-induced and glutamine-repressed cultures. *Molecular and General Genetics 211,* 35-40.

***Hurlburt, B.K.,* and C. Yanofsky** (1990) Enhanced operator binding by *trp* superrepressors of *E. coli.* *J. Biological Chemistry* ***265,* 7853-7858.**

Hurlburt, B.K.,* and C. Yanofsky** (1992) The NH_2-terminal arms of E. *coli trp* repressor participate in repressor/operator association. *Nucleic Acids Research* ***20, 337-341.

***Hurlburt, B.K.,* and C. Yanofsky** (1992) The *trp* repressor/trp operator interaction: equilibrium and kinetic analysis of complex formation and stability. *J. Biological Chemistry 267,* 16783-16789.

Hurlburt, B.K.,* and C. Yanofsky** (1993) Analysis of heterodimer formation by the E. *coli trp* repressor. *J Biological Chemistry* ***268, 14794-14798.

Czernik, P.J., Shin, D. S. and *Hurlburt, B.K.* (1994) Functional selection and characterization of DNA binding sites for the *E. coli trp* repressor. *J. Biological Chemistry* ***269,*** 27869-27875.

Drake, R.R. and *Hurlburt, B.K.* (1996) Synthesis and uses of photoactive DNA. in *Photoaffinity Labelling: Methods and Applications. Volume II.- Photoactive DNAIRNA. p.* 13-20. RP International, Mt. Prospect, IL.

Czernik, P.J., Peterson, C.A. and *Hurlburt, B.K.* (1996) Preferential binding of MyoD/E12 versus Myogenin/E12 to the MSV enhancer *in vitro. J. Biological Chemistry 271,* 9141-9149.

Stebbins, M.A., Hoyt, A.M., Jr., Sepaniak, M.J., and *Hurlburt, B.K.* (1996) Design and optimization of a capillary electrophoretic mobility shift assay involving *trp* repressor/DNA complexes. *J Chromatog.* ***683,*** 77-84.

Maleki, S.J. and *Hurlburt, B.K.* (1997) High-level expression and rapid purification of Myogenin, MyoD and E12. *Protein Expression and Purification 9,* 91-99.

Maleki, S.J., Royer, C.A. and *Hurlburt, B.K.* (1997) MyoD-E12 heterodimers and MyoD-MyoD homodimers are equally stable. *Biochemistry, 36,* 6762-6767.

Czernik, P.J., McDermott, P.F., and *Hurlburt, B.K.* (submitted to I *Bacteriology)* Functional selection and initial characterization of seventy new binding sites for *trp* repressor in the E. *coli* genome.

Mackintosh, S.G., McDermott, P.F. and *Hurlburt, B.K.* (in revision, *Molecular Microbiology)* Mutational analysis of the NH_2 terminal arm of *trp* repressor indicates a multifunctional domain.

Maleki, S.J., Royer, C.A. and *Hurlburt, B.K.* (in preparation for submission to *Biochemistry)* Energetics of enhancer binding by MyoD, Myogenin and E12 *in vitro.*

C. Research Support.

Completed Research Support

Grant #GM47264 (R29) 05/01/1992 – 04/30/1998
National Institute of General Medical Sciences
Structure/function of *trp* repressor of *E. coli.*
Major goal: Characterize the mechanism of repressor assembly and binding.
Role: PI

Pending Research Support

Grant #GM47264 Competitive Renewal. 04/01/1998 – 03/31/2003
National Institute of General Medical Sciences
Structure/function of *trp* repressor of *E. coli.*
Major goal: Characterize the mechanism of repressor assembly and binding.
Role: PI

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Tammy M. Lutz-Rechtin	Research Associate
eRA COMMONS USER NAME	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Hendrix College, Conway, AR	B.A.	1987-1991	Chemistry
University of Arkansas for Medical Sciences	Ph.D.	1991-1995	Biochem/Mol. Bio.

A. Positions and Honors.

Positions

- Fall 1997-Present: Research Associate, University of Arkansas for Medical Sciences, Biochemical Research, Department of Biochemistry and Molecular Biology.
- 1996-Fall 1997:Instructor/Research Scientist, University of Arkansas for Medical Sciences, Arkansas Cancer Research Center, Department of Otolaryngology-Head and Neck Surgery.
- Fall 1995-1996: Postdoctoral Fellow/Research Scientist, University of Arkansas for Medical Sciences, Arkansas Cancer Research Center, Department of Otolaryngology -- Head and Neck Surgery.
- Fall 1991-1995: Graduate Assistant, University of Arkansas for Medical Sciences, Department of Biochemistry and Molecular Biology.
- Summer 1991: Lab technician, University of Arkansas for Medical Sciences, Department of Biochemistry and Molecular Biology.
- Summer 1990-1991: Student independent study research, Hendrix College, Department of Chemistry.

Honors

- College of Medicine Student Research Grant,"Characterization of HSV-1 TK." $2,000; University of Arkansas for Medical Sciences, September. 1993-94.
- UAMS Student Travel Award, "Characterization of HSV-1 Thymidine Kinase Utilizing Nucleoside and Nucleotide Photoaffinity Analogs." $500; American Society for Virology Meeting, Madison, WI, July 1994.
- American Society of Virology Student Travel Award, "Characterization of HSV-1 Thymidine Kinase Utilizing Nucleoside and Nucleotide Photoaffinity Analogs." $500; American Society for Virology Meeting, Madison, WI, July 1994.

B. Selected peer-reviewed publications (in chronological order).

***Rechtin, T.M.,* and Dornhoffer, J.D.** (1997) CD44v6 expression in the Ear Canal: Specificity for Cholesteatoma within the Middle Ear*. In preparation*.

***Rechtin, T.M.,* Flock, S.T., and Dornhoffer, J.D.** (1997) Exclusive Expression of the E48 Antigen in Middle Ear Cholesteatomas. *Submitted for publication*.

***Rechtin, T.M.,* Hermonat, P.L., Farris, P., Vural, E., McGhee, M.E., and Stem, S.** (1997) High Prevalence of Human Papillomaviruses in Laryngeal Squamous Cell Carcinomas and Adjacent Normal Mucosa. *In preparation.*

***Rechtin, T.M.,* Stem, S., Farris, P., and Hermonat, P.L.** (1997) Association Of Adeno-Associated Virus and Human Papillomaviruses in Laryngeal Squamous Cell Carcinomas. *Submitted for publication*.

***Rechtin, T.M.,* Dornhoffer, J.D., Drake, R.R., and Flock, S.T.** (1997) Photoinimunotherapy of Squamous Epithelial Diseases: A New Technique. *Manuscript in Preparation*.

Drake, R.R., McMasters, R., Krisa, S., Hume, S.D., *Rechtin, T.M.,* Saylors, R.L., Chiang, Y.W., Govindarajan, R., and Munshi, N.C. (1997) Metabolism and Activities of 3'-azido-2',3'-dideoxythymidine and 2',3'-didehydro-2',3'-dideoxythymidine in Herpesvirus Thymidine Kinase transduced T-lymphocytes. Antiviral Research, 35(3), 177-185.

Hermonat, P.L., Han, L., Wendel, P.J., Quirk, J.G., Stem, S., Lowery, C.L., and *Rechtin, T.M.* (1997) Human papillomaviruses DNA is elevated in first trimester spontaneously aborted products of conception compared to elected specimens. *Virus Genes*, 14(1), 13-17.

Drake, R., Hume, S., Black, M.E., and *Rechtin, T.M.* (1996) Analysis of the thymidylate and ganciclovir binding domains of HSV-1 and HSV-2 TKs. *Submitted for publication*.

***Rechtin, T.M.,* Sunthanker, P., Wade, D., Endling, S., and Drake, R. R.** (1996) Analysis of mutant HSV-1 TK binding sites utilizing bisubstrate nucleotides and photoaffinity analogs. *Submitted for publication*.

***Rechtin, T.M.,* Black, M., and Drake, R.R.** (1996) Proteolytic Mapping of the Thymidine/Thymidylate Binding Site of Herpes Simplex Virus Type I Thymidine Kinase: A General Photoaffinity Labeling Method for Identifying Active Site Peptides. *Analytical Biochem*., 237, 135-140.

Black, M.E., *Rechtin, T.M.,* and Drake, R.R. (1996) Effect on Substrate Binding of an Alteration at the Conserved Aspartate-162 in Herpes Simplex Virus Type I Thymidine Kinase. *J. Gen. Virol.*, 77:1521-1527.

Mao, F., *Rechtin, T.M.,* Jones, R., Cantu, A., Anderson, S., Radominska, A., Moyer, M.P. and Drake, R.R. (1995) Synthesis and Biochemical Properties of 5-Azido-3'-Azido-2',3'-dideoxyuridine: A Photoaffinity Analog of 3'-Azidothymidine. *J. Biol. Chem.*, 270, 13660-13664.

***Rechtin, T.M.,* Black, M.E., Mao, F., Lewis, M.L. and Drake, R.R.** (1995) Purification and Photoaffinity Labeling of Herpes Simplex Virus Type-1 Thymidine Kinase. *J Biol. Chem.*, 270, 7055-7060.

Batchu, R.B., Miles, D.A., *Rechtin, T.M.,* Drake, R.R. and Hermonat, P.L. (1995) Cloning, Expression and Purification of Full Length Rep 78 of Adeno-Associated Virus as a Fusion Protein in *Escherichia coli. Biochem. Biophys. Res. Comm.*, 208,714-720.

Paul, P., *Lutz, T.M.,* Osborn, C., Kyosseva, S., Elbein, A.D., Towbin, H., Radominska, A., and Drake, R.R. (1993) Synthesis and Characterization of a New Class of Membrane-Associated UDPGlycosyltransferases Inhibitors. *J. Biol. Chem*., 268, 12933-12938.

C. Research Support.

Completed Research Support

None.

Pending Research Support

None.

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

The PI and Co-Investigator have a combined total of 1650 sq. ft. of laboratory space in the Biomedical Research Center at the University of Arkansas for Medical Sciences. Laboratories are located within 200 ft. of each other. The laboratories are collectively equipped for all bacteriological, biochemical, immunological and recombinant DNA techniques. Common equipment areas include all major equipment required.

Clinical:

Not applicable.

Animal:

Not applicable.

Computer:

Office and laboratory computers are linked via the university LAN to the DEC Alpha in the campus Computer Services office, MEDLINE, and to the Alpha4000 with GCG software in the Co-Investigator's laboratory.

Office:

The PI's office is located approximately 100 ft. from the laboratory. The Co-Investigator's office is located the same distance from his laboratory and approximately 300 ft. from the PI's office.

Other:

Both departments include systems for the production of high-quality (18 megaohm) distilled water, as well as common facilities for dishwashing and sterilization. The BRC includes an electronics repair shop.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Major equipment items within the Department of Microbiology and Immunology and the Department of Biochemistry and Molecular Biology include an automated film processor, an oligonucleotide synthesizer (Perceptive Expedite), automated DNA sequencer (ABI Model 377), several high-speed and ultracentrifuges, biosafety and laminar-flow hoods, bacterial incubators/shakers, gamma counters, ultralow freezers, UVNis spectrophotometers and an image documentation system capable of storing images in a format appropropriate for the computer-driven, quantitative analysis of autoradiograph images.

RESEARCH PLAN

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.

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.

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).

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.

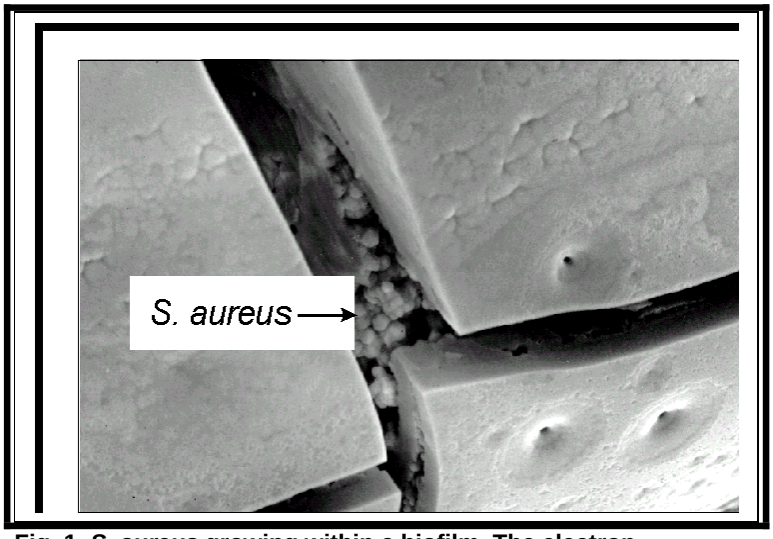

Fig. 1. *S. aureus* growing within a biofilm. The electron micrograph was prepared from the bone of an experimentally-infected rabbit (18).

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* P_3 promoter may be dependent on the *S. aureus* stress-response sigma factor σ^B. 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.

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). 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).

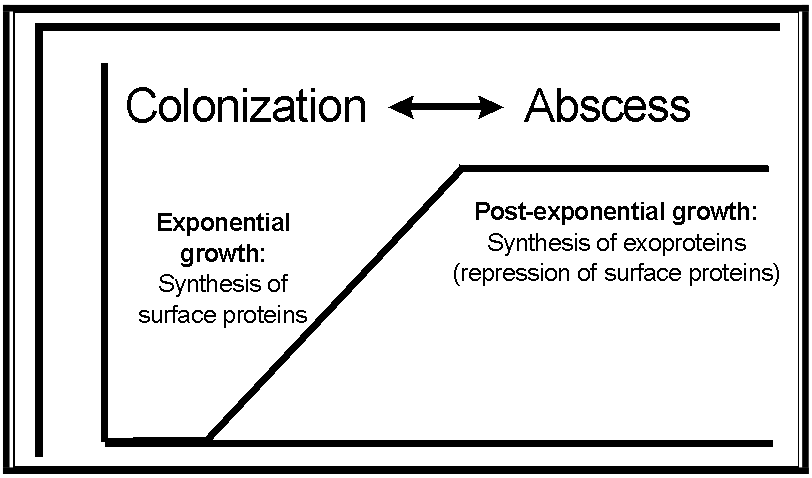

Fig. 2. Schematic representation of *S. aureus* phenotypic switching *in vitro* and its potential relevance to growth *in vivo*.

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. Iandolo, 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.

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

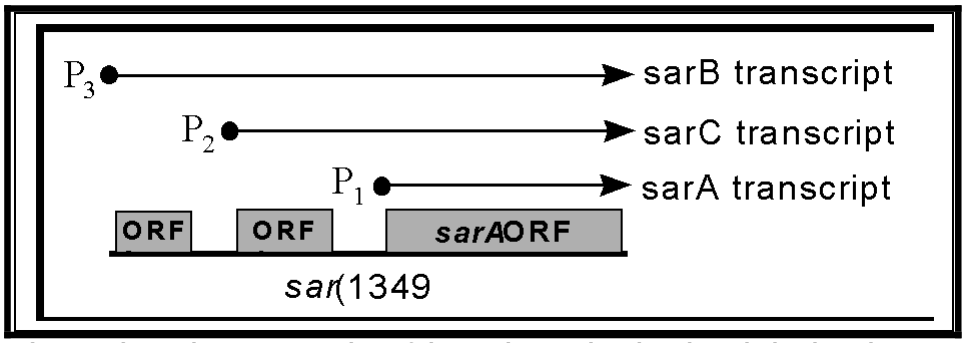

Fig. 3. Schematic representation of the *sar* locus showing the relative location and size of each transcript. The P3, P2 and P1 promoters (filled circles) were defined by Bayer et al. (2). Shaded boxes indicate open-reading frames (ORFs). ORF3 and ORF4 potentially encode peptides with 18 and 39 amino 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* P_2 and P_3 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).

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* P_2 and P_3 promoters (27).** The following discussion is intended to emphasize the significance of that observation.

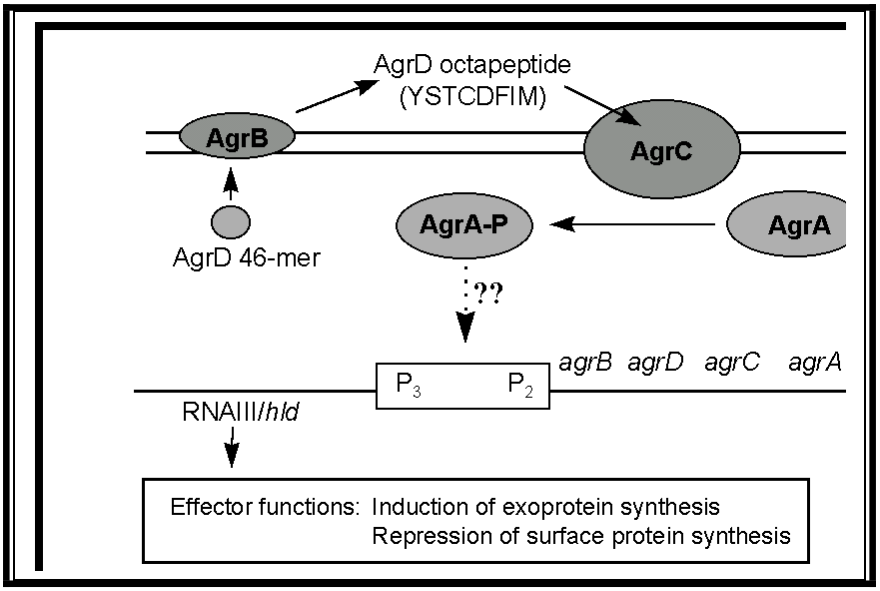

Fig. 4. Schematic representation of the *agr* regulatory system. The agrBDCA operon encodes a "quorum-sensing" system (see text) that modulates production of the RNAIII effector molecule. The function of each component and the uncertainty associated with the interaction between phorphorylated AgrA and the P_2/P_3 promoter region are discussed in the text.

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 (RNAII) from a promoter designated P_2. 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 (P_3) located ~120 bp upstream of P_2 (27). The P_3 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).**

The interaction between SarA and agr. The scenario described above suggests that AgrA is a DNA-binding protein that activates transcription from the *agr* P_2 and P_3 promoters. However, attempts to demonstrate that AgrA binds *cis* elements upstream of the *agr* P_2 or P_3 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* P_2 and P_3 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* P_2 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* P_3 promoter is required for the induction of RNAIII synthesis and the regulated expression of the *agr* target genes encoding alpha-**

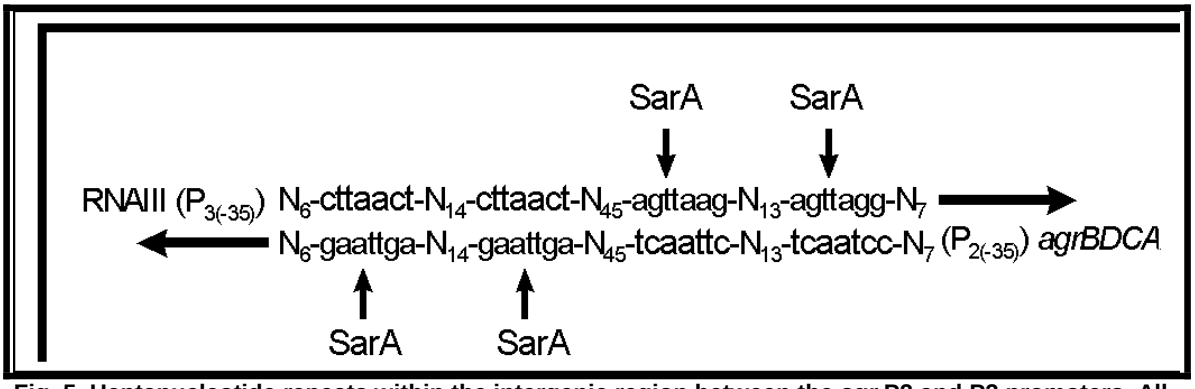

Fig. 5. Heptanucleotide repeats within the intergenic region between the *agr* P2 and P3 promoters. All heptanucleotide sites are indicated as potential SarA-binding sites although the stochiometry of SarA binding is unknown.

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* P_3 promoter and as an imperfect repeat (AGTTAGG) upstream of the *agr* P_2 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 P_2 promoter have an orientation opposite to those upstream of the P_3 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.

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).

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).

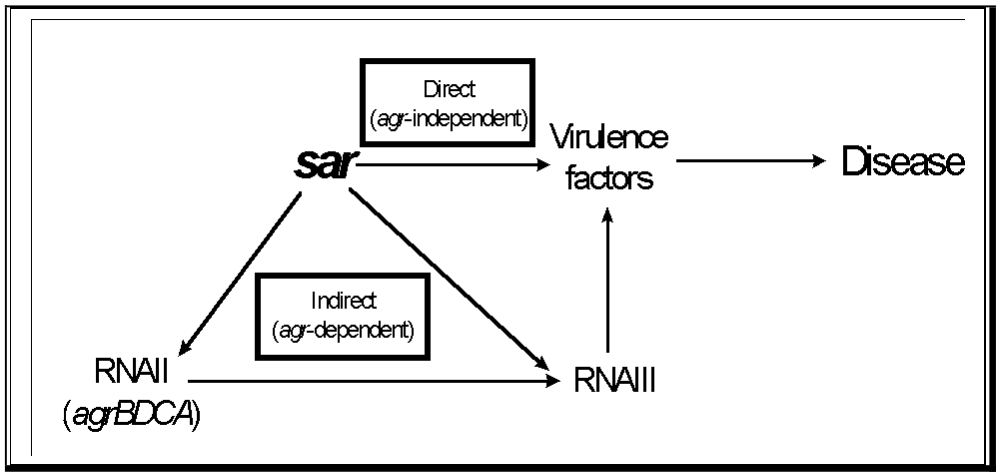

Fig. 6. Summary of the impact of *sar* on expression of *S. aureus* virulence factors. The *agr*-dependent pathway is designated "indirect" because the effect on expression of the target gene is mediated through an intermediary, presumably RNAIII. The *agr*-independent pathway is designated "direct"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

PRELIMINARY RESULTS

This proposal is a collaboration between the PI and Dr. Barry Hurlburt in the Department of Biochemistry and Molecular Biology. The collaboration takes advantage of the expertise of the PI in the molecular genetics of *S. aureus* and the biochemical expertise of Dr. Hurlburt in transcription factor structure and function (14,15). The overall goals are 1) correlation of the expression of the sarA, sarB and sarC transcripts with the production and activity of SarA, 2) characterization of the mechanism by which *sar* regulates expression of the *S. aureus* collagen adhesin gene (*cna*) and 3) identification and characterization of additional *S. aureus* genes

under the direct regulatory control of SarA. We have assembled all of the experimental tools required to accomplish these objectives. Specifically, we have (i) purified SarA in a form capable of binding an appropriate DNA target, (ii) generated an affinity-purified antibody against purified SarA, (iii) constructed a *xylE* reporter plasmid that can be used to assess the functional activity of SarA (Specific Aim #1) and define the sequence characteristics required for the regulation of *cna* transcription (Specific Aim #2), (iv) cloned the regions encoding the sarA, sarB and sarC transcripts for use in complementation experiments, (v) demonstrated that SarA binds a DNA target upstream of *cna* and begun the process of localizing the SarA binding site and (vi) obtained or generated *sar* and *agr* mutants in both *cna*-positive and *cna*-negative *S. aureus* strains. The experiments done to accomplish each of these tasks are described in detail below.

Cloning and expression of sarA. The polymerase chain reaction (PCR) was used to amplify the *sarA* coding region from *S. aureus* strain RN6390. Utilizing *Nde*I and *Bam*HI restriction sites incorporated into the oligonucleotide primers, the fragment containing the *sarA* coding region was cloned into the *E. coli* expression vector pET9A. Because the *Nde*I site (CATATG) in the vector overlaps an ATG start codon, cloning of the *sarA* coding region into the *Nde*I site places the *sarA* structural gene in perfect register with the vector-derived ribosome binding site. Recombinant proteins are therefore expressed as full-length, wild type proteins without fusions to exogenous peptide or protein tags. After cloning the *sarA* PCR fragment into pET9A and confirming the identity of the cloned fragment by DNA sequencing (data not shown), the recombinant plasmid (pETSarA) was used to transform *E. coli* strain BL21(DE3)pLysS. Transformants were grown to mid-log phase before

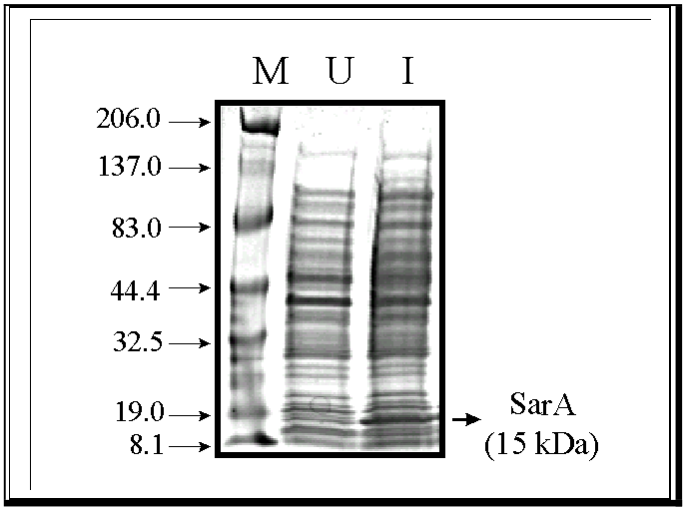

Fig. 7. Expression of SarA in *E. coli*. Whole cell lysates were resolved using 10-20% SDS-PAGE gradient gels. Lane designations: M, molecular weight markers (sizes indicated in kDa); U, uninduced; I, induced.

inducing SarA expression by adding IPTG to a final concentration of 0.4 mM. After two hours, cells were harvested and lysed by sonication. The presence of SarA in the crude lysate was confirmed by SDS-PAGE followed by Coomassie Brilliant Blue staining (Fig. 7).

Purification of SarA. A 500 ml culture of the BL21(DE3)pLysS *E. coli* strain containing pETSarA was induced and lysed as described above. After removing the insoluble material in the crude lysate by centrifugation, the soluble fraction was subjected to a series of ammonium sulfate precipitations culminating at 70% saturation. The pellet from each precipitation was resuspended in SDS-PAGE buffer and examined along with an aliquot of the supernatant (Fig. 8, left). The supernatant remaining after the final precipitation was found to contain ~70% SarA.

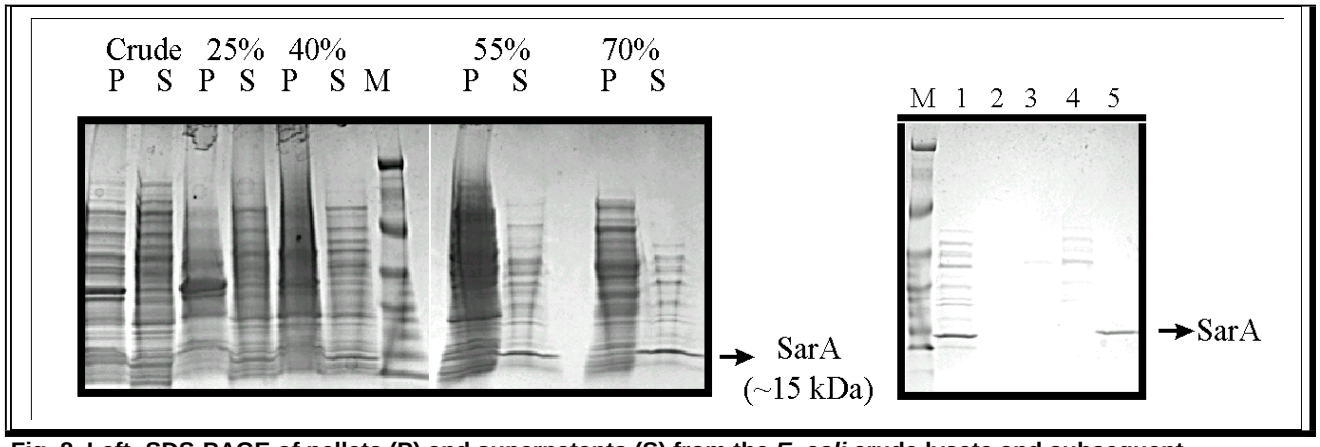

Fig. 8. Left: SDS-PAGE of pellets (P) and supernatants (S) from the *E. coli* crude lysate and subsequent ammonium sulfate precipitations. The lane marked "M" contains the same molecular weight markers shown in Fig.7. Right: Purification of SarA. Lane 1 contains an aliquot of the supernatant from the 70% ammonium sulfate precipitation. Lanes 2 and 3 contain fractions of the "flow-through" obtained after applying the 70% supernatant to the heparin sepharose column. Lanes 4 and 5 contain elution fractions obtained by increasing the salt concentration of the elution buffer. Fraction 5 contained SarA in the absence of detectable levels of other proteins.

SarA was subsequently purified to apparent homogeneity by ion exchange chromatography using Heparin-Sepharose (Fig. 8, right).

Confirmation that the purified protein is SarA. To ensure that the protein purified from our *E. coli* lysates is SarA, we performed electrophoretic mobility shift assays (EMSA) with a 45 bp DNA fragment containing the heptad repeats *cis* to the *agr* P_3 promoter (see Fig. 5, Background and Significance). The 45 bp fragment was generated by

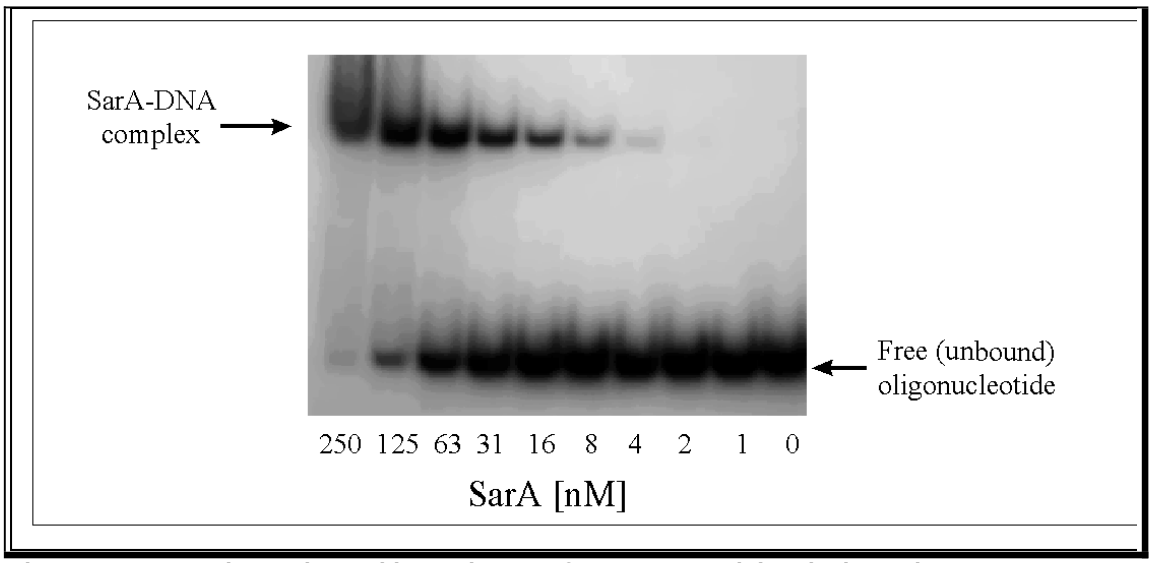

Fig. 9. EMSA experiment done with a 45 bp DNA fragment containing the heptad repeats upstream of the *agr* P3 promoter and purified SarA. Lane designations indicate the concentration of SarA in each binding reaction. All lanes contain an equal amount (~10 pmole) of ^{32}P-labeled DNA.

synthesizing and annealing complementary oligonucleotides. After labeling the target DNA with ^{32}P, the fragment was allowed to equilibrate in solution with varying amounts of the purified protein. The mixture was then resolved by native gel electrophoresis. The fact that a mobility shift was observed with the *agr*-derived target DNA (Fig. 9) confirms that the protein we purified from the *E. coli* lysates is SarA. **Moreover, these results, together with the results of our EMSA experiments employing DNA fragments derived from the region upstream of *cna* (see below), demonstrate that our *E. coli*-derived SarA preparation is appropriate for the experiments aimed at the identification of additional SarA targets within the *S. aureus* genome (Specific Aim #3).**

Generation of SarA-specific antiserum. To generate affinity-purified anti-SarA antibodies, two rabbits were immunized by sequential subcutaneous injections of 1) 75 µg SarA suspended in Freund's Complete Adjuvant (day -35), 2) 75 µg SarA suspended in Freund's Incomplete Adjuvant (day -20) and 3) 150 µg SarA without adjuvant (day -5). To test for seroconversion, rabbits were bled from the ear vein on day -5 and the sera tested by enzyme-linked immunosorbent assay (ELISA) using microtiter plates coated with purified SarA and goat anti-rabbit IgG conjugated to horseradish peroxidase. One of two rabbits was found to have antibody reactive against SarA (data not shown). The seropositive rabbit was subsequently bled by cardiac puncture on day 0. SarA antibody present in the antiserum was purified by affinity chromotography using CNBr-activated sepharose coupled to purified SarA. The specificity of the antibody will be determined by blotting cell lysates prepared from wild-type strains with lysates prepared from *sar* mutants (Table 1) and by EMSA supershift assays using purified SarA and an appropriate DNA target. The affinity-purified antibody preparation will be used in Western blot assays (Specific Aim #1) and for the immunoprecipitation of SarA-DNA complexes (Specific Aim #3).

Construction of xylE reporter fusion vector. A plasmid containing a promoter-less *xylE* reporter gene was obtained from Dr. Ken Bayles at the University of Idaho. The plasmid was constructed by amplifying the *xylE* structural gene using oligonucleotide primers containing engineered *Hind*III and *Bam*HI restriction sites in the 5' and 3' primers respectively. These restriction sites were used to clone the amplification product into pUC19. We used the same restriction sites to subclone the promoter-less *xylE* gene from pUC19 into the *E. coli-S. aureus* shuttle vector pLI50 (data not shown). By introducing DNA fragments containing the *cis* elements associated with the *agr* P_2 and P_3 promoters upstream of the promoter-less *xylE* gene, this construct can be used to assess the activity of SarA as a transcriptional activator (Specific Aim #1). Additionally, by introducing DNA fragments corresponding to the DNA upstream of *cna* (P_{cna}), the *xylE* reporter fusion can be used to define the sequence requirements necessary for the SarA-mediated regulation of *cna* transcription (Specific Aim #2).

Studies with the S. aureus collagen adhesin gene (cna). Recent studies in our laboratory have focused on the role of collagen binding in the pathogenesis of staphylococcal osteomyelitis. These studies have led to the following conclusions. **First**, we have established that the collagen-binding capacity (CBC) of *S. aureus* is a direct function of the presence and expression of *cna* (21) and that *cna* is encoded within a discrete chromosomal

element that is not present in most *S. aureus* isolates (19,39). The *cna* element does not encode any additional genes and its presence does not disrupt a gene present in *cna*-negative strains (19). These results are relevant to this proposal because they established that 1) a fragment containing ~500 bp of the DNA upstream of *cna* is sufficient for the regulated expression of *cna* from a pLI50 construct (20) and 2) the regulatory loci that control *cna* transcription are present in both *cna*-positive and *cna*-negative strains (20). **Second**, as discussed in the Background and Significance section of this proposal, we have established that the expression of *cna* is regulated by *sar* in an *agr*-independent manner (20, 21). Specifically, when we examined the collagen binding capacity (CBC) of *cna*-positive strains that carry mutations in the *sar* and/or *agr* loci, we found that mutation of *agr* results in only a slight increase in CBC that is not apparent in all strains (20) while mutation of *sar* results in a dramatic increase in the ability to bind collagen (Fig. 10) and a corresponding increase in *cna* transcription (Fig. 11). Moreover, mutation of both *sar* and *agr* had an additive effect on CBC and *cna* transcription. **Because these results would not be expected if the regulatory effects of *sar* were a function of the SarA-dependent activation of *agr* transcription, we conclude that *sar* is the primary regulatory element controlling expression of *cna* and that the regulatory effects of *sar* on *cna* transcription are independent of the regulatory impact of *sar* on *agr*.**

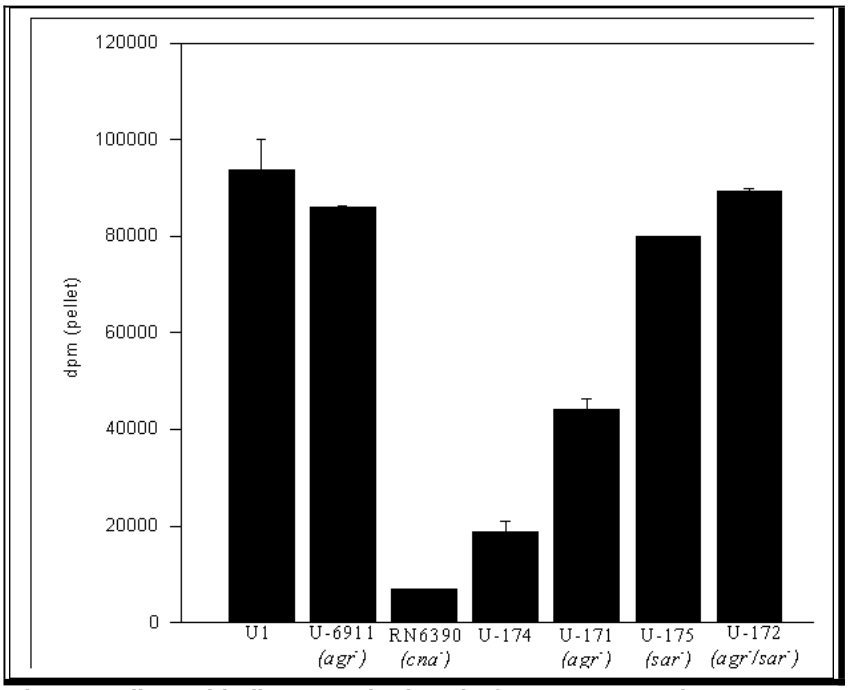

Fig. 10. Collagen binding capacity (CBC) of RN6390 *sar* and *agr* mutants. The prefix "U" designates a UAMS strain (Table 1). UAMS-1 is a clinical isolate that encodes and expresses *cna*. U-6911 is an *agr*-null mutant of U-1. Collagen binding assays were done using ^{125}I-labeled collagen as previously described (20).

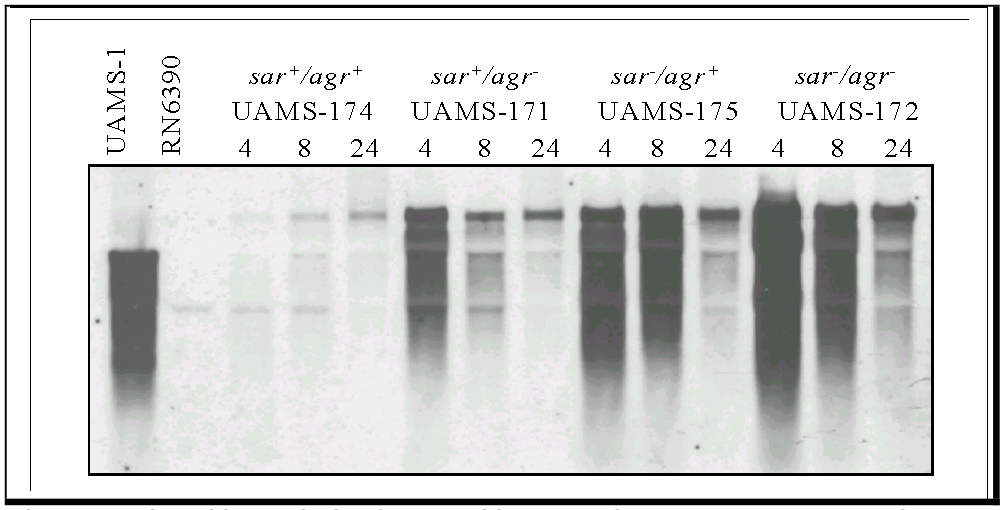

Fig. 11. Northern blot analysis of *cna*-positive *sar* and *agr* mutants. RNA samples taken at various time points (top) were blotted using a *cna* probe. Growth curves indicate that the transition between exponential and post-exponential growth occurred between the 4 and 8 hr time points (data not shown). UAMS-1 encodes a *cna* gene with a single B domain and was included as a positive control. RN6390 does not encode *cna* and was included as a negative control. The *cna* gene introduced into RN6390 and the RN6390 *sar* and *agr* mutants is derived from FDA574 and includes three B domains; as a result, the *cna* mRNA in these strains is larger than the *cna* mRNA in UAMS-1.

Studies on the mechanism of sar-mediated regulation of cna transcription. To determine whether the regulation of *cna* transcription is a direct function of SarA or is due to an unidentified regulatory factor under the regulatory control of SarA, we generated a series of short (~140 bp) overlapping fragments that collectively span the region extending 360 bp region upstream of the *cna* coding region.

EMSA experiments done with purified SarA and each of these fragments suggests that at least one SarA-binding site exists within ~200 bp of the *cna* start codon (Fig. 12). Although sequence analysis of the DNA region upstream of *cna* reveals three sites that match the heptanucleotide *agr* target at 6 of 7 bp (19), it is important to note that, by comparison to the repeats upstream of the *agr* promoters, none of these sites are appropriately placed with respect to the putative *cna* promoter or with respect to each other (19, 30). These results suggest that the SarA-binding sites upstream of the *agr* promoters may be relatively unique by comparison to other SarA targets. Whether the distinction between the SarA-binding sites upstream of *agr* and the SarA-binding sites upstream of *cna* is related to the fact that SarA-binding results in the activation of *agr* transcription and the repression of *cna* transcription is one of the issues that will be addressed during the course of these studies. **Most importantly, we believe our preliminary results regarding the *sar*-mediated regulation of *cna* transcription provide us with a unique system by which we can investigate the *agr*-independent branch of the *sar* regulatory pathway.**

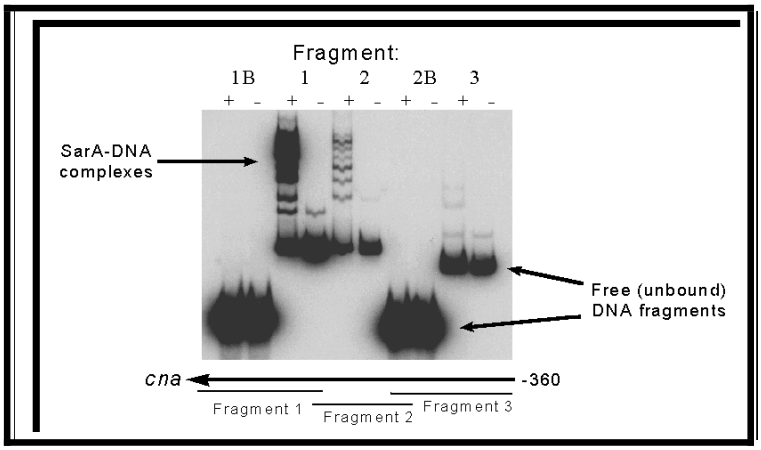

Fig. 12. EMSA done with DNA fragments derived from the region immediately upstream of *cna* (19) and purified SarA. The presence (+) or absence (-) of SarA is indicated above each lane. All binding assays were done with a constant amount of ^{32}P-labeled DNA (10 pmole) and 55 nM SarA. Fragments 1, 2 and 3 collectively span the 360 bp region upstream of the *cna* start codon as indicated below the figure. Fragments 1B and 2B are extraneous PCR products obtained during the amplification of fragments 1 and 2 respectively. To ensure binding specificity, both fragments were purified and included in the EMSA experiments.

Cloning of the DNA regions encoding the sarA, sarB and sarC transcripts. We used PCR to amplify individual DNA fragments corresponding to the regions encoding the sarB, sarC and sarA transcripts. All three fragments were amplified using an antisense primer that corresponds to the region downstream of a *rho*-independent terminator ownstream of *sarA* (2). The 5' primers used for each amplification were designed to result in PCR products that include all potentially relevant DNA sites associated with the *sar* P_1, P_2 and

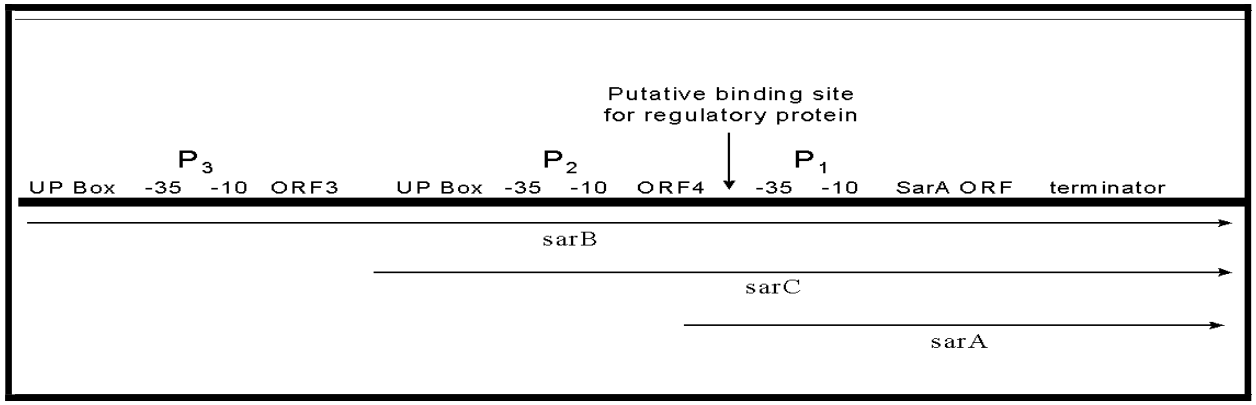

Fig. 13. Molecular architecture of the *sar* locus as defined by Bayer et al. (2). DNA sites with the potential to impact transcription are indicated above the bold line. Thin arrows below the bold line indicate the relative location of the DNA fragments amplified and cloned for use in our complementation studies.

P_3 promoters (Fig. 13).

Specifically, Bayer et al. (2) identified a potential “UP box” (an A/T-rich DNA site that may facilitate binding of the RNA polymerase subunit) upstream of the *sar* P_2 and P_3 promoters. The region upstream of the *sar* P_1 promoter also contains what Bayer et al. (2) identified as a putative binding site for regulatory DNA-binding proteins (Fig. 13). Although no function for these DNA sites has been demonstrated, it nevertheless seemed prudent to include each site within the respective PCR product. Therefore, the 5' end of the largest fragment (1288 bp) is located 20 bp upstream of the UP box adjacent to the *sar* P_2 promoter while the 5' end of the second fragment (978 bp) is located 16 bp upstream of the UP box adjacent to the *sar* P_3 promoter. These fragments span the regions encoding sarB and sarC respectively (2). The 5' end of the smallest fragment (708 bp) is located 226 bp upstream of the *sarA* start codon and includes the region encoding both the putative binding site for regulatory DNA-binding proteins (Fig. 13). The relatively long 5’ extension was chosen because the region extending 189 bp upstream of the sarA transcript appears to be required for efficient transcription of the genes encoded within *agr* (6). Each amplification product was cloned using the TOPO-TA vector (Invitrogen, Carlsbad, CA) and subcloned into the pLI50 shuttle vector. By introducing each of these plasmids into a *cna*-positive *S. aureus sar* mutant (see below), we can confirm the results of our experiments correlating the production of each *sar* transcript with the production of SarA and the temporal pattern of *cna* transcription (Specific Aim #2).

Construction of S. aureus sar mutants. We have generated all combinations of *sar* and *agr* mutants in both *cna*-positive and *cna*-negative *S. aureus* strains. Specifically, we obtained two *sar* mutants (Sar R and 11D2) and their corresponding wild-type parent strains (RN6390 and DB respectively) from Dr. Ambrose Cheung at Rockefeller University. The *cna* gene was introduced into Sar R and RN6390 by 11-mediated transduction from CYL574 (21). This transduction results in the integration of *cna* into the chromosomally-encoded lipase gene (*geh*). The RN6390 (*geh::cna*) derivative has been designated UAMS-174 while the Sar R (*geh::cna*) derivative has been designated UAMS-175. The successful introduction of *cna* was confirmed by Southern blot (data not shown) and by demonstrating that, unlike the RN6390 and Sar R parent strains, UAMS-174 and UAMS-175 bind collagen (Fig. 10). To generate *sar/agr* double mutants, we used 11-mediated transduction to introduce the *agr*-null mutation from RN6911 into Sar R, UAMS-174 and UAMS-175. RN6911 is an RN6390 strain in which the entire *agr* locus including the region encoding RNAIII has been replaced with *tetM*. Importantly, the *geh::cna* insertion in UAMS-174 and UAMS-175 confers tetracycline resistance by virtue of the *tetK* gene. That is relevant because, while both *tetK* and *tetM* confer tetracycline-resistance, only *tetM* confers resistance to minocycline.

Table 1. *S. aureus agr* and *sar* mutants.

Strain	Relevant genotype	Source/Comments
RN6390	*agr*$^+$, *sar*$^+$	*S. aureus* 8325-4. Dr. Ambrose Cheung, Rockefeller University.
Sar R	*agr+, sar-*	RN6390 *sarA* mutant. Dr. Ambrose Cheung.
RN6911	*agr-, sar+*	RN6390 *agr*-null mutant. Dr. Richard Novick, New York University.
UAMS-173	*agr-, sar-*	Transduction from RN6911 into Sar R.
UAMS-174	*agr+, sar+, cna+*	Transduction from CYL574 into RN6390.
UAMS-175	*agr+, sar-, cna+*	Transduction from CYL574 into Sar R.
UAMS-171	*agr-, sar+, cna+*	Transduction from RN6911 into UAMS-174.
UAMS-172	*agr-, sar-, cna+*	Transduction from RN6911 into UAMS-175.
DB	*agr+, sar+*	*S. aureus* wild-type strain. Provided by Dr. Ambrose Cheung.
11D2	*agr+, sar-*	DB *sarA* mutant. Dr. Ambrose Cheung.

It was therefore possible to select for the *agr*-null mutation by plating transductants on medium containing 2 µg/ml minocycline. The same selection was used to identify the *agr*-null derivative of Sar R. The *agr*-null derivatives of UAMS-174, UAMS-175 and Sar R were designated UAMS-171, UAMS-172 and UAMS-173 respectively (Table 1).

The mutations in each of the strains described in Table 1 were confirmed at the genotypic level by Southern blot (data not shown) and at the phenotypic level by collagen binding assays (Fig. 10) and Northern blot (Fig. 11). The availability of these strains will be important in the experiments aimed at 1) correlating the production of each of the *sar* transcripts with the production of functional SarA (Specific Aim #1), 2) complementation analysis aimed at defining the *sar* transcripts required to restore control of *cna* transcription (Specific Aim #2) and 3) confirmation of the *sar*-mediated regulatory control of additional *S. aureus* target genes (Specific Aim #3).

RESEARCH DESIGN AND METHODS

Specific Aim #1: Defining the relationship between sar transcription and the production of SarA.

Rationale. Northern blot analysis of the *sar* locus reveals the presence of three transcripts (sarA, sarB and sarC), all of which share a common termination site and include the entire *sarA* structural gene (2). Each transcript is produced from its own promoter in a growth-phase dependent manner. Specifically, expression of the sarA and sarB transcripts is highest during exponential growth while expression of sarC is highest during the post-exponential growth phase (2). In our experience with clinical isolates of *S. aureus*, the growth-phase dependency of sarB expression is much tighter than the growth-phase dependency of sarA expression (i.e. sarB is not made during post-exponential growth while sarA is made is reduced amounts by comparison to exponential phase levels). **The overall objective of these experiments is to correlate the temporal production of the sarA, sarB and sarC transcripts with the production and activity of SarA.** The experiments include assays for the direct detection of SarA (Part A) as well as assays capable of assessing SarA function (Parts B and C). The direct detection of SarA will be done by Western blot using the affinity-purified anti-SarA antibody preparation discussed in Preliminary Results. We will correlate the results of these experiments with functional assays assessing the activity of SarA as a DNA-binding protein (Part B) and the activity of SarA as a transcriptional activator (Part C). Establishing the correlation between the accumulation of SarA and the functional activity of SarA will allow us to address the possibility that the small ORFs contained within the sarB and sarC transcripts serve a functional role with respect to SarA activity. The activity of SarA as a DNA-binding protein will be assessed by EMSA experiments performed with whole cell lysates and DNA fragments that contain the heptad repeats upstream of the *agr* P_2 and P_3 promoters. The function of SarA as a transcriptional activator will be assessed using fusions between each of the *agr* promoters and a *xylE* reporter gene. Because these experiments will employ fusions with the *agr* promoters, the experiments will specifically address the function of SarA as a transcriptional activator. Similar experiments utilizing fusions between the *xylE* reporter and the *cna* promoter region (P_{cna}) will allow us to correlate the results with the functional activity of SarA as a transcriptional repressor

(Specific Aim #2).

Experimental design. The Western blot and EMSA experiments (Parts A and B respectively) will be done using cell lysates from *S. aureus* strains RN6390, DB, Sar R, 11D2 and RN6911. RN6390 and DB are wild-type strains that encode and express all three *sar* transcripts (7,11). The inclusion of both strains is based on the observation that, by comparison to DB, the *sarA* gene in RN6390 has a nonsense mutation that results in the deletion of 11 amino acids from the C-terminus of the SarA protein (2). Although Bayer et al. (2) suggest that this truncation has no effect on the production or activity of SarA, the phenotype of RN6390 *sar* mutants (e.g. Sar R) is somewhat distinct by comparison to a DB *sar* mutant (e.g. 11D2) (9,11). Based on that, we believe the inclusion of both strains will allow us to make a more a comprehensive assessment of the functional activity of SarA in *S. aureus*. It is anticipated that SarA will be detected at some point in the growth cycle of RN6390 and DB, with one of the experimental questions being whether the temporal accumulation and/or activity of SarA in RN6390 differs from the pattern observed in DB. Sar R and 11D2 are *sar* mutants generated in RN6390 and DB respectively. These strains carry transposon insertions in the *sarA* structural gene and are included as negative controls for their respective parent strains. The inclusion of RN6911 in these experiments requires comment. RN6911 is an RN6390-derived *agr*-null mutant in which the entire *agr* locus (including the region encoding RNAIII) has been replaced with a tetracycline-resistance determinant (in *tetM*) (38). Its inclusion is based on the hypothesis that RNAIII may contribute to the production of SarA. That hypothesis requires explanation since it has been demonstrated both in our laboratory and by other investigators that mutation of *agr* does not affect the production of any *sar* transcript (7). However, we believe the inclusion of RN6911 is warranted because 1) the impact of *agr* on *sar* has not been examined at any level other than Northern blot analysis of *sar* transcription in *agr* mutants (6,23) and 2) the *agr*-encoded RNAIII molecule is known to affect the production of at least one *S. aureus* protein (alpha-toxin) at the post-transcriptional level (26). Importantly, the post-transcriptional effect of RNAIII on alpha-toxin production involves an "anti-attenuation" in which translation of the *hla* mRNA is enhanced in the presence of RNAIII. **In the absence of RNAIII**, the *hla* mRNA transcript forms a stem-loop structure that sequesters the ribosome-binding site within the duplexed stem region (26). The unavailability of the ribosome-binding site results in the translational attenuation of alpha-toxin production. **In the presence of RNAIII**, this attenuation is relieved due to the formation of an RNAIII:*hla* mRNA duplex molecule in which the ribosome-binding site on the *hla* mRNA is exposed and available for translation (26). The observation that this "anti-attenuation" involves a short (~80 bp) region of the RNAIII molecule that exhibits only partial complementarity (~75%) with *hla* mRNA suggests that a similar effect may occur with the transcripts encoding other proteins including SarA. It should be re-emphasized that the Northern blot analysis of *sar* transcription in *agr* mutants does not address the possibility that RNAIII has an effect on the production of SarA from an existing *sar* transcript. Based on that, the second experimental question to be addressed by these studies is whether RN6911 produces SarA and, if so, whether the temporal pattern of SarA production is affected by the absence of RNAIII.

A. Determination of the temporal accumulation of SarA by Western blot.

Methods. To quantitate the accumulation of SarA, whole cell lysates will be prepared from each strain using cells taken at various stages of *in vitro* growth. Specifically, each strain will be grown in tryptic soy broth at 37^{o}C with constant aeration. Two-liter cultures will be used to allow for the removal of relatively large volumes (~500 ml) at each time point. It is anticipated that relatively large volumes may be required to compensate for the relatively low cell density during the exponential growth phase. Cells will be harvested after 2, 4, 6 and 8 hours of growth. Under these growth conditions, the transition between exponential and post-exponential growth can be anticipated to occur between the 4 and 6 hr time points. Additionally, based on our Northern blot analysis (data not shown), these time points encompass each of the growth phases associated with maximal production of the sarA, sarB and sarC transcripts. In this as well as all other time course experiments, our assumptions about growth phase will be verified by monitoring the optical density of each culture at 560 nm. Because the mutations in Sar R, 11D2 and RN6911 involve chromosomal insertions, all cultures will be grown without antibiotic selection. However, to ensure the stability of each insertion, duplicate plate counts utilizing medium with and without antibiotics will be done at the completion of each experiment.

Lysates will be prepared according to the method of Mahood and Kahn (25). Briefly, cells will be harvested by centrifugation, washed and resuspended in 4.0 ml TEG buffer (25 mM Tris-Cl (pH 8.0), 5 mM EGTA). The cell suspension will be subjected to two freeze-thaw cycles prior to adding KCl to a final concentration of 0.15 M and lysostaphin to a final concentration of 0.2 mg/ml. After incubating on ice for 45 min., the cell suspension will be subjected to two additional freeze-thaw cycles and then centrifuged at 30,000 rpm in a Beckman SW40 rotor. After harvesting the supernatant, glycerol will be added to a final concentration of 20% (v/v). The supernatant will then be dialyzed against a buffer consisting of 10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 50 mM $NaCl_2$ and 20% (v/v) glycerol. After determining the protein concentration using the Bradford protein assay (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA), aliquots of each lysate will be resolved by SDS-PAGE (10-20% gradient gels) and the resolved proteins transferred to nitrocellulose membranes. Western blot analysis will be performed using the Phototope-HRP Western Blot Detection Kit (New England Biolabs, Beverly, MA). Briefly, the nitrocellulose membrane will be sequentially incubated in 1) blocking buffer consisting of Tris-buffered saline containing 0.1% Tween-20 and 5% gelatin, 2) an appropriate dilution of the affinity-purified anti-SarA antibody preparation (see below) and 3) goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP). Detection of the HRP-conjugated antibody will be done using the LumiGlo chemiluminescent substrate and exposure to X-ray film. The use of a radiographic images will allow us to quantitate signal strengths by densitometric analysis.

To determine the appropriate dilution of the anti-SarA antibody preparation, Western blot analysis will be performed as described above using purified SarA. In the first set of experiments, a constant amount of SarA (~500 ng) will be resolved in multiple lanes of the same gel and then blotted using varying dilutions of the antibody. In the second set of experiments, the dilution of antibody that results in a reproducible, strong signal in the first experiment will be tested against decreasing amounts of purified SarA. If the sensitivity of the assay is sufficient (estimated at 10 ng of purified SarA), subsequent Western blots will be done using the same dilution of antibody. If the detection limit is >10 ng, then we will decrease the antibody dilution to bring the concentration of SarA antibody up to

level capable of detecting lesser amounts of protein. Alternatively, we can scale up the procedure for production of cell lysates to bring the final yield of SarA into the detectable range. In either case, the sensitivity of the Western blot protocol will be confirmed by repetitive blots using IgG purified from pre-immune serum obtained from the same rabbit used for the SarA immunization. The IgG fraction will be purified by protein G-sepharose affinity chromatography. The dilution of the SarA antibody to be used in subsequent experiments will be the concentration of antibody that yields a reproducible signal with the least amount of purified SarA without a background signal with the same dilution of pre-immune serum. Once the appropriate dilution is determined, a standard curve will be constructed based on the densitometric analysis of Western blot radiographs. The amount of SarA in each cell lysate will be determined by comparison to this standard curve.

The amount of SarA in lysates prepared from each strain at different time points will be correlated with the production of individual *sar* transcripts in the same strains at the same time points. The *sar* transcripts will be detected by Northern blot using a *sarA*-specific gene probe (because all three transcripts include the entire *sarA* gene, all three can be detected with equal efficiency using the same gene probe). Protocols for the isolation of RNA, resolution of RNA by agarose gel electrophoresis, and the detection of mRNA's using digoxygenin-labeled DNA probes and the Lumigen chemiluminescent substrate (Boehringer Mannheim, Indianapolis, IN) are all well-established in the PI's laboratory (20, 22).

B. Determination of the temporal accumulation of SarA by EMSA.

Methods. The DNA-binding activity of SarA will be assessed using the DB, 11D2, RN6390, Sar R and RN6911 lysates produced for our Western blot experiments. The EMSA experiments will be done using the 45-bp DNA fragment corresponding to the *agr* P_3 promoter region (see Preliminary Results) and a similar fragment corresponding to the *agr* P_2 promoter region. The inclusion of both DNA targets is based on the results of Heinrichs et al. (23), who suggested that SarA exhibits differential binding to the *agr* P_2 and P_3 promoters. The concentration of DNA to be used in each set of experiments will be determined based on equilibrium dissociation constants (K_D) determined in EMSA experiments using varying concentrations of purified SarA and each of the DNA targets (13, 14). Specifically, the amount of DNA used in EMSA experiments with whole cell lysates will be 100-fold higher than the K_D determined using purified SarA. Under those conditions, stoichiometric binding occurs such that the concentration of protein required to bind 50% of the available DNA ($P_{1/2}$) is equal to 50% of the input DNA concentration [DNA_o] (5). For example if the K_D is 200 pM, then we will use 20 nM of input DNA. Based on the conversion formula $P_{1/2}$ = 1/2 [DNA_o], the point at which 50% of the DNA (10 nM) is bound will indicate that the extract contains 10 nM of SarA capable of binding an appropriate DNA target.

Once the appropriate concentration of each DNA target is determined, EMSA experiments will be done using a constant amount of ^{32}P-labeled DNA and various dilutions of the whole cell lysate prepared from each strain at each time point. Bound and unbound DNAs will be resolved by native gel electrophoresis and the unbound fraction quantitated by laser densitometry. The bound fraction, determined by subtracting the unbound fraction from the total input DNA, will be plotted against the volume of crude extract used in the mixture. The specificity of the protein-DNA complex observed by EMSA will be confirmed by supershift with

anti-SarA antibody and by competition experiments employing unlabeled DNAs. The amount of SarA capable of binding DNA will be expressed in moles of SarA per volume of extract and will be compared with the accumulation of each of the *sar* transcripts and the accumulation of SarA as defined by the Western blot protocol described in Specific Aim #1, Part A. To determine whether the DNA-binding activity of SarA is a direct function of the concentration of SarA in the cell, the results will be reported as the ratio of the total amount of SarA as defined by Western blot (Specific Aim #1, Part A) versus the amount of SarA bound to each DNA target (e.g. [total SarA/bound SarA]). Because an increase in the DNA-binding activity of SarA will be reflected by a decrease in this ratio, any decrease will be taken as an indication that the DNA-binding activity of SarA can be affected by post-translational modifications. Correlating such changes with the production of specific *sar* transcripts will allow us to assess the possibility that the short ORFs encoded within the sarB and sarC transcripts are important for SarA function. Finally, by correlating the results obtained with a DNA fragment corresponding to the *agr* P_2 promoter region with the results obtained with a DNA fragment corresponding to the *agr* P_3 promoter region, any functional difference in the ability to bind the two *agr* promoters can be assessed.

C. Determination of the temporal accumulation of SarA by transcriptional activation.

Methods. To confirm the results of our experiments correlating the production of each of the *sar* transcripts with the production of SarA and the activity of SarA as a DNA-binding protein, we will also assess the growth-phase dependent activity of SarA as a transcriptional activator. These experiments will also allow us to determine whether transcriptional activation from the *agr* P_2 and P_3 promoters occurs at the same time and under the same conditions. These experiments will be done using deriviatives of RN6390, Sar R, DB, 11D2 and RN6911 carrying either a P_2-*xylE* reporter fusion or a P_3-*xylE* reporter fusion. The reporter vector was constructed by inserting a promoter-less *xylE* gene downstream from the multiple cloning site in the pLI50 *E. coli-S. aureus* shuttle vector (see Preliminary Results). To generate the appropriate fusions, we used PCR to amplify the entire 186-bp region between the RNAII and RNAIII transcription start sites. This amplification was done with primers that contain engineered restriction sites incorporated for use in cloning into the pLI50-*xylE* vector. Specifically, *Hind*III restriction sites were added at both the 5' and 3' ends of the amplified fragment. The presence of these restriction sites will allow us to clone the 186-bp fragment upstream of the *xylE* reporter gene in both orientations. Because the heptad repeats upstream of the P_2 and P_3 promoters are directional, this approach should allow us to assess activation from each promoter while at the same time allow for any influence of the distal binding sites on activity from the proximal promoter. To select for the *xylE* reporter plasmid, 5 µg/ml chloramphenicol will be added to the growth medium. Variants of each strain that contain the promoter-less *xylE* vector will be included as negative controls.

Catechol 2,3-dioxygenase (CATase) activity will be assessed using cellular lysates prepared from samples taken at the same time points described for Specific Aim #1, Parts A and B. Our ability to correlate CATase activity with the SarA-mediated activation of transcription from the P_2 and P_3 promoters is based on the observations that (i) mutation of *sar* in RN6390 results in reduced production of both RNAII and RNAIII and (ii) the production of RNAII and RNAIII is restored when a clone carrying the *sarA* structural gene is introduced into the RN6390 *sar* mutant (6). These results clearly indicate that transcription from the P_2 and P_3 promoters in RN6390 is at least partially dependent on SarA. CATase assays will be done essentially as described by Ray et al. (34). Briefly, 10 ml samples will be harvested from each culture at 2 hr intervals and the cells harvested by centrifugation. Cell pellets will be washed with 2 ml of 20

mM potassium phosphate buffer (pH 7.2) and then frozen overnight at -70°C. Pellets will then be thawed, resuspended in 2 ml of APEL buffer (100 mM potassium phosphate (pH 7.5), 20 mM EDTA, 10% acetone, 200 µg lysostaphin per ml), and incubated for 30 min. at 37°C. After adding 20 µl Triton X-100 and incubating on ice for an additional 30 min., extracts will be centrifuged to pellet the cellular debris. CATase assays will be done by mixing 250 µl of each cell extract with 2.75 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 0.2 mM catechol. All reactions will be carried out at 30°C (34, 44). Results will be monitored spectrophotometrically by measuring the absorbance at 375 nm (17). Under these conditions, 1 milliunit of catechol 2,3'-dioxygenase activity corresponds to the formation of 1 nmol of 2-hydroxymuconic semialdehyde per minute (44).

To ensure that the results reflect the functional activity of SarA, the CATase activity observed when the *xylE* fusions to the *agr* P_2 and P_3 promoters are introduced into the Sar R and 11D2 mutants will be taken as the background CATase activity associated with each of the parent strains. The background activity observed with each mutant will be subtracted from the results obtained with the corresponding parent strain. The results will then be compared with the values obtained for experiments assessing the accumulation of SarA as defined by Western blot analysis (Specific Aim #1, Part A) and the DNA-binding activity of the accumulated SarA as defined by EMSA (Specific Aim #1, Part B). If the activity of SarA as a transcriptional activator is dependent only on the production of SarA, then changes in CATase activity as a function of growth phase should be consistent with changes in the amount of SarA. An increase in CATase activity without a corresponding increase in the concentration of SarA will be taken as an indication that SarA exists in alternative forms that differ in their ability to activate transcription. Comparison of the results obtained with the *agr* P_2 and P_3 promoters will allow us to assess whether such differences are promoter-dependent. Correlation of the results with the production of different *sar* transcripts will provide an indication of whether the small *sar* ORFs are required for SarA activity.

Specific Aim #2: Characterization of the mechanism by which sar regulates expression of the S. aureus collagen adhesin gene (cna).

Rationale. Expression of the *S. aureus* collagen adhesin gene (*cna*) is highest during the exponential growth phase and falls to almost undetectable levels during post-exponential growth (20).To assess the regulatory impact of *sar* and *agr* on *cna* transcription, we introduced *cna* into RN6390, Sar R and RN6911. Comparison of *cna*-positive derivatives of each strain indicates that *sar* is the primary regulatory element controlling expression of *cna* and that the regulatory impact of *sar* is independent of the interaction between SarA and *agr* (20,21). Subsequent experiments indicate that the *sar*-mediated regulation of *cna* transcription involves a direct interaction between SarA and *cis* elements upstream of *cna* (see Preliminary Results, Fig. 12). **Importantly, these results represent the first indication that SarA binds any DNA target other than the heptad repeats upstream of the *agr* P_2 and P_3 promoters.** Moreover, the SarA-mediated regulation of *cna* transcription differs from the SarA-mediated regulation of *agr* transcription in two respects. First, mutation of *sar* results in the increased expression of *cna* (21) and the decreased expression of RNAII and RNAIII (23). Second, the DNA upstream of *cna* does not contain heptad repeats like those associated with the *agr* promoters (19, 30). Based on these differences, we believe it is important that the mechanism of the *sar*-mediated regulation of *cna* transcription be addressed in detail. That is the overall objective of Specific Aim #2.

Experimental design. The experiments in this section are divided into three areas. The first is directed toward identification of the specific *sar* transcripts required to restore the *sar*-mediated

regulation of *cna* transcription. These experiments will be done by introducing plasmids encoding each *sar* transcript into a *cna*-positive *sar* mutant and assessing the effect on *cna* transcription. The results will be correlated with the production of each *sar* transcript from each clone (it should be emphasized that the *sar* transcripts overlap such that the sarB transcript is all inclusive while the sarC transcript includes the promoter for the sarA transcript; see Preliminary Results, Fig. 13). Additionally, once the SarA-binding site upstream of *cna* has been defined (see below), EMSA experiments will be done using cell lysates from *sar* mutants complemented with each *sar* transcript and the appropriate DNA target. These experiments will allow us to correlate the results of our complementation analysis with the production and activity of SarA. The second set of experiments can be divided into three phases, all of which are directed toward identification and characterization of the SarA-binding site upstream of *cna*. Specifically, we will 1) localize the SarA binding site using EMSA experiments done with purified SarA and DNA fragments derived from the region upstream of *cna*, 2) identify and partially characterize the binding site within the target fragment by DNA footprinting and methylation interference assays and 3) characterize the sequence-specificity of the binding site using EMSA experiments done with mutagenized DNA targets and purified SarA. Importantly, all of the protocols required to complete the Specific Aim #2, Part B experiments are well-established in the Co-I's laboratory (14,15). The final set of experiments in this section is directed toward assessing the *in vivo* significance of SarA binding. These experiments will be done using transcriptional fusions between sequence variants of the *cis* elements upstream of *cna* and a promoter-less *xylE* reporter gene.

A. Complementation of the defect in *cna* transcription observed in *sar* mutants.

Methods. The experimental approach taken in these experiments is based on studies demonstrating that complementation of different defects in *sar* mutants requires the introduction of DNA fragments encoding different *sar* transcripts (23). As a first step toward defining the mechanism by which *sar* regulates expression of *cna*, we will attempt to complement the defect in *cna* transcription using plasmid-borne DNA fragments encoding each *sar* transcript (see Preliminary Results, Fig. 13). Each of these fragments has already been cloned into the pLI50 *E. coli-S. aureus* shuttle vector. Each clone will be introduced into the *cna*-positive, *sar* mutant UAMS-175. Transformation will be done by electroporation as previously described (20). Transformants will be selected by plating on tryptic soy agar containing 5 µg per ml chloramphenicol. After verifying the presence of the appropriate plasmid, transformants will be grown in tryptic soy broth and sampled at 2 hr intervals. Transformants will be characterized with respect to (i) restoration of the expected *sar* transcripts, (ii) the temporal pattern of *sar* transcription, (iii) the capacity to bind collagen and (iv) the temporal pattern of *cna* transcription. Northern blot analysis will be done as previously described (22) using probes corresponding to *sarA* and *cna*. As discussed above, the fact that all three *sar* transcripts include the *sarA* gene will allow us to detect all three transcripts using the same *sarA* probe. The *cna* gene probe was generated by PCR using genomic DNA from *S. aureus* strain UAMS-1 as template (20). Collagen-binding assays will be done using ^{125}I-labeled type I collagen as previously described (20). Restoration of the wild-type phenotype with respect to each of the parameters noted above will be made by comparison to the *cna*-positive, *sar*-positive strain UAMS-174 (see Preliminary Results, Table 1). As the experiments aimed at defining the SarA-binding site upstream of *cna* progress (see below), we will correlate the results of these experiments with the accumulation of SarA as determined by Western blot (see Specific Aim #1, Part A) and by EMSA experiments performed with the appropriate DNA fragment and purified SarA.

B. Characterization of the SarA DNA-binding site upstream of *cna*.

Methods #1: Localization of the SarA-binding site upstream of cna. EMSA experiments employing a series of short, overlapping fragments derived from the region upstream of *cna* and purified SarA indicate that at least one SarA-binding site exists within approximately 200 bp upstream of the *cna* start codon (see Preliminary Results, Fig. 12). To further localize the SarA-binding site(s) upstream of *cna*, we will synthesize DNA fragments corresponding to progressively smaller regions of the DNA upstream of *cna* and use these fragments in EMSA experiments utilizing purified SarA. Appropriate fragments will be generated either by synthesizing and annealing complementary pairs of oligonucleotides or by PCR. The fact that we have already sequenced the region extending 930 bp upstream of the *cna* transcriptional start site (19) will greatly facilitate the synthesis of the appropriate DNA targets. EMSA experiments will be done using standard procedures (5). Briefly, ^{32}P-labeled DNA fragments will be mixed with purified SarA diluted to concentrations ranging from 1 to 100 nM (this concentration range was chosen based on preliminary experiments demonstrating a band shift with 55 nM SarA). After an appropriate incubation period, SarA-DNA complexes will be resolved by native gel electrophoresis as illustrated in Preliminary Results. It is anticipated that these experiments will allow us to localize the SarA-binding site to a region spanning 50-100 bp. However, we recognize that SarA-binding sites may exist across a relatively long stretch of DNA with intervening and perhaps irrelevant nucleotides in between each site. To address that issue, all fragments that are bound by SarA will be used in “mix and match” competition experiments aimed at defining the relative affinity of SarA for different binding sites. The experiments described below will be done starting with the DNA fragments that exhibit the highest affinity for SarA (i.e. that exhibit a gel shift with the lowest concentration of SarA and are not competitively inhibited in the presence of other, unlabeled DNA fragments).

Methods #2: Mapping of the SarA-binding site upstream of cna. Once the smallest DNA fragment(s) bound by SarA have been identified, the same fragment(s) will be characterized by DNase I footprinting and methylation interference assays. Both sets of experiments will be done with purified SarA and ^{32}P-labeled DNA fragments. **For DNaseI footprinting**, SarA will be diluted to an appropriate concentration (based on the results of the EMSA experiments described above) in an assay buffer consisting of 10 mM Tris-Cl (pH 8.0), 5 mM MgCl2, 1 mM CaCl2, 100 mM KCl, 2 mM dithiothreitol (DTT), 50 µg/ml bovine serum albumin (BSA) and 2 µg/ml calf thymus DNA. After allowing the mixture to equilibrate, DNase I diluted in assay buffer without BSA or calf thymus DNA will be added and the exposure allowed to proceed for 2 minutes. The appropriate concentration of DNase I will be determined empirically (4). A control tube without DNase I will also be included in all experiments. Reaction products will be examined by polyacrylamide gel electrophoresis and exposure to X-ray film according to standard protocols (4). **For methylation protection experiments**, labeled DNA fragments will be incubated for 5 minutes at room temperature with dimethyl sulfate (DMS) diluted in an assay buffer consisting of 50 mM sodium cacodylate (pH 8.0) and 50 mM EDTA. The reaction will be stopped by adding 1.5 M sodium acetate containing 1 M 2-mercaptoethanol. After ethanol precipitation (1), the methylated DNA will be mixed with purified SarA and the mixture allowed to equilibrate for 15-30 minutes at room temperature. The SarA-DNA complexes will then be resolved using native polyacrylamide gels. The bands corresponding to SarA-DNA complexes and to free DNA will be purified and subjected to piperidine cleavage. The reaction products will then be resolved using polyacrylamide sequencing gels. After exposure to X-ray film, protected bases will be detected by comparison to the free DNA lane; fragments corresponding to protected bases will be absent in the lane derived from the SarA-DNA complexes (1). It is anticipated that the DNase I footprinting will allow us to define the region containing the SarA-binding site and that the methylation interference assays will provide an indication of the relative

significance of specific guanine and adenine residues within the protected region.

Methods #3: Defining the sequence characteristics of the SarA-binding site upstream of cna. The final set of experiments in this section (Specific Aim #2, Part B) is directed toward confirmation of the SarA-binding site(s) and identification of the specific sequence requirements necessary for SarA binding. Specifically, once we have identified the protected region in our footprinting experiments, we will generate sequence variants by synthesizing complementary pairs of oligonucleotides that span the protected region. The same approach was taken with the 45-bp fragment synthesized for the preliminary EMSA experiments done to confirm the identity of our purified SarA (Fig. 9). However, in this case, we will design the oligonucleotides to introduce specific nucleotide substitutions within the protected region. If the protected region does, in fact, represent a specific SarA target, then it should be possible to generate variants that are not bound by SarA. Although it is not possible to anticipate the nature or extent of all substitutions, the methylation interference assays should provide some information with regard to critical adenine and/or guanine residues. Moreover, it should be emphasized that the Molecular Biology Core Facility at the PI's institution is located in the Co-I's laboratory and includes an oligonucleotide synthesizer that can be used to generate as many sequence variants as necessary. The results will be evaluated by comparing the sequence of DNA fragments that are bound by SarA with the sequence of DNA fragments that are not bound by SarA. To more accurately assess the relative affinity of SarA for different DNA targets, all experiments will be done using SarA at various concentrations. The specificity of binding will subsequently be confirmed by repeating the EMSA experiments using combinations of labeled and unlabeled target DNAs. Taking this approach, it should be possible to identify those base pairs that are necessary for SarA binding (i.e. those base pairs that cannot be changed without eliminating the SarA DNA-binding target).

C. Correlation of SarA binding with regulation of *cna* transcription.

Methods. The functional significance of the putative SarA-binding site(s) will be assessed by *in vivo* experiments in which different versions of the *cna* target(s) are cloned into the *xylE* reporter gene vector and introduced into *S. aureus*. The activity of different mutagenized versions of the binding site(s) will be assessed by performing CATase assays using cells taken at various stages of growth and comparing the results to CATase assays done using the *xylE* reporter construct containing the wild-type binding site. To reduce interference from an endogenous binding site, these experiments will be done in a *cna*-negative strain of *S. aureus* (e.g. RN6390). The specific DNA fragments to be introduced into the *xylE* reporter vector will be determined based on the results of the EMSA and footprinting experiments decribed in Part B. Most of the other methods required for these experiments have already been described. Specifically, the *xylE* reporter vector, the methods to be employed for the synthesis of different versions of the SarA target upstream of *cna*, and the CATase assays to be used to assess expression from different versions of the SarA-binding site are all described in other sections of this proposal. However, it should be emphasized that we do not intend these experiments to be as all inclusive as those described in Section B. More specifically, the objective of these experiments is to confirm that the putative SarA binding site identified in Part B is functional in terms of serving as a SarA target in *S. aureus*. Based on that, we will only examine a restricted set of fragments using the *xylE* reporter vector. Specifically, we will identify a subset of fragments that retain the ability to bind SarA and a subset of fragments that have lost the ability to bind SarA and will then clone these fragments into the promoter-less *xylE* vector. To introduce the target region into the *xylE* vector, appropriate restriction site linkers will be added to each fragment using standard procedures. Plasmids carrying different variants of the SarA target(s) upstream of *cna* will be introduced into RN6390 by electroporation. The results will be

evaluated by comparison to the results obtained with a *xylE* fusion containing the wild-type promoter region and will be reported as the ratio of CATase activity observed with each variant versus the activity observed with the wild-type sequence (CATase activity of variant/CATase activity of wild-type). Because mutation of *sar* results in overexpression of *cna*, elimination of the SarA binding site should result in increased *xylE* expression. For that reason, any increase in this ratio will be taken as an indication that the putative SarA-binding site is functional *in vivo* in *S. aureus*.

Specific Aim 3: Identification of S. aureus virulence factors under the direct control of SarA.

Rationale. We believe the preceding discussion establishes the fact that *sar* modulates expression of *S. aureus* genes through both *agr*-dependent and *agr*-independent regulatory pathways. In most cases, the existence of the *agr*-independent pathway can only be inferred from the comparison of *sar* and *agr* mutants. On the other hand, our results with regard to the regulation of *cna* transcription establish the existence of additional SarA targets within the *S. aureus* genome and suggest that the regulation of other genes that are differentially regulated by *sar* and *agr* may also involve a direct interaction between SarA and *cis* elements upstream of the target genes. Additionally, the characterization of *sar* mutants has been limited to a relatively small set of genes defined by the availability of appropriate gene probes and/or quantitative phenotypic assays. For that reason, our understanding of the *agr*-independent pathway is incomplete both in terms of the mechanism by which *sar* regulates expression of different target genes and the scope of SarA targets. However, the increasing size of the *S. aureus* genomic database and the availability of computer programs capable of the detailed analysis of megabase quantities of DNA have set the stage for detailed studies aimed at the direct identification of genes under the direct regulatory control of SarA. Our successful purification of SarA in a form capable of binding diverse DNA targets (e.g. *cis* elements upstream of the *agr* and *cna* promoters), together with the established expertise of the Co-I in the use of PCR-assisted binding site selection (PCR-ABS) (15), places us in a unique position with regard to the identification of additional SarA targets within the *S. aureus* genome. Finally, the molecular expertise of the PI in the molecular biology of *S. aureus* and the successful production of appropriate *sar* and *agr* mutants will allow us to confirm the results of our PCR-ABS experiments by directly testing for *sar*-mediated regulation of the genes *cis* to putative SarA-binding sites. The ability to pursue these experiments to completion will be greatly facilitated as information regarding the complete nucleotide sequence of the *S. aureus* genome becomes available.

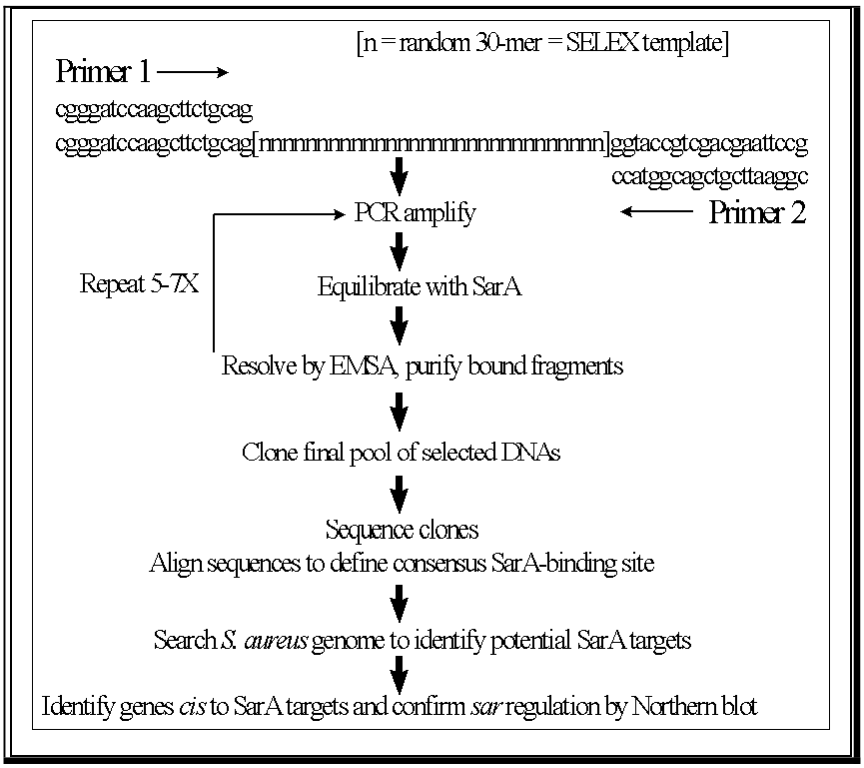

Fig. 14. Flow-chart for for PCR-assisted binding site selection (PCR-ABS).

Experimental design. The PCR-ABS technology is based on the synthesis of a complex pool of DNAs that contain a random sequence across a target region large enough to bind the transcription factor of interest (15). Based on our experience with other bacterial transcription factors (15), as well as the data of Morfeldt et al. (27) suggesting that SarA binds to a DNA target contained within a 28 bp region defined by heptanucleotide repeats, we have chosen to utilize a 30 bp target region for our first round of experiments. Synthesis of the appropriate pool of DNAs is accomplished by synthesizing three oligonucleotides (Fig. 14). One of these will be a 70-mer that includes defined 20 bp sites at each end. The only requirements for the design of these defined sites are 1) that they have no obvious similarity to the binding site of interest and 2) that they are distinct by comparison to each other. The first of these requirements is somewhat difficult to predict since the consensus binding site for SarA has not been defined (indeed, that is the point of these experiments). At present, all we can do is design the 20 bp sites such that they do not contain any form of the *agr* heptads (AGTTAAG, AGTTAGG, CTTAACT, and CCTAACT) or any sequence longer than a few base pairs (~5-6) that matches any part of the sequence upstream of *cna*. The second requirement (that the 20 bp sites be unique with respect to each other) is important because the PCR-ABS technology utilizes PCR to generate a random sequence across the region between the 20 bp sites (Fig. 14). It is therefore important that the 20 bp sites not contain sequence elements that would allow the primers to anneal to each other. The sequence of the 30 bp region between the 20 bp sites is irrelevant (see below). The identity of the 2nd and 3rd oligonucleotides required for PCR-ABS is based on the identity of the 20 bp sites at each end of the 70-mer. Specifically, one of these (primer 1) is identical to the 20 bp site at one end of the 70-mer while the other (primer 2) is complementary to the 20 bp site at the other end of the 70-mer (Fig. 14). The complementary primer is used in an initial extension to generate a double-stranded DNA molecule. The two primers are then used together to amplify the 70 bp fragment. Importantly, this amplification is done using an equimolar mixture of all four nucleotides such that the 30 bp region between the primer sites has a random sequence. The PCR-ABS technology is based on binding of the protein of interest to this region of random sequence. The basic steps in the PCR-ABS protocol are 1) amplification of a pool of fragments containing random sequences across the 30 bp target site, 2) EMSA experiments in which the pool of fragments is allowed to bind to the transcription factor of interest (e.g. SarA), 3) purification of bound fragments and 4) cloning and sequencing of fragments selected for the ability to bind SarA (Fig. 14). Once a sufficient number of bound fragments have been identified, they are sequenced and the 30 bp regions aligned to reveal the

consensus SarA-binding site. An example of such an alignment is shown below (Fig. 15). Once the consensus binding site is established, its presence within the *S. aureus* genome will be assessed by computer-assisted homology searches. In those cases in which a potential binding site is appropriately placed with respect to a potential *S. aureus* promoter, the *sar*-mediated regulation of the gene *cis* to this binding site will be examined by the Northern blot analysis of wild-type strains and their corresponding *sar* mutants. Importantly, the PCR-assisted binding site selection protocol, which is also known by various other acronyms (e.g. SELEX), is well-established in the Co-I's laboratory as evidenced by the successful identification of *trp* repressor binding sites within the *E. coli* genome (15).

	-10	**-9**	**-8**	**-7**	**-6**	**-5**	**-4**	**-3**	**-2**	**-1**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
G	2	96	4	4	0	0	0	100	4	23	9	47	33	22	51	76	17	25	4	41
A	4	4	2	96	0	0	100	0	0	51	2	43	6	0	37	10	10	53	4	26
T	43	0	79	0	0	100	0	0	87	11	8	6	18	78	7	5	63	13	0	9
C	51	0	15	0	100	0	0	0	9	15	81	4	43	0	5	10	10	10	93	24
	C	***G***	***T***	***A***	***C***	***C***	***A***	***G***	***T***	***A***	***C***	***G***	***C***	***T***	***G***	***G***	***T***	***A***	***C***	***G***

Fig. 15. Derivation of a consensus sequence for a DNA-binding protein by PCR-ABS. The top row indicates the relative position of each base. The numbering scheme is based on the *trp* operator sequence and reflects the fact that the binding site is symmetrical. Each possible bases is indicated along the left with the numbers in each cell indicating the percentage of clones that contain the corresponding base at each position. The consensus sequence (defined as the most likely base to be found at each position across the entire target region) is indicated along the bottom.

Methods #1: Selection of SarA-binding targets. The success of the experiments described in Specific Aim #3 is dependent on the efficiency of our selection for SarA-binding targets. We have chosen the PCR-ABS approach based on the successful utilization of this technology by the Co-I for the characterization of binding sites for the *E. coli trp* repressor (15). The three DNA fragments required to implement the protocol (Fig. 14) were synthesized using an oligonucleotide synthesizer in the Molecular Biology Core Facility at the University of Arkansas for Medical Sciences. The sequence of the 20 bp termini of the 70 bp oligonucleotide and the corresponding 20 bp primers is illustrated in Fig. 14. These sequences were chosen because 1) their utility as PCR primers has already been confirmed by the Co-I in his analysis of *trp* repressor-binding sites (15), 2) each primer includes three restriction sites that can be used to clone selected DNAs (Fig. 16) and 3) neither primer has significant similarity to DNA sequences upstream of the *agr* and *cna* promoters. Based on these characteristics, it should be possible to amplify appropriate target DNAs without introducing an "artificial" SarA-binding site. The central region of the template DNA will be randomized and simultaneously labeled using a mixture of the four bases that includes [^{32}P]dCTP. To generate the starting

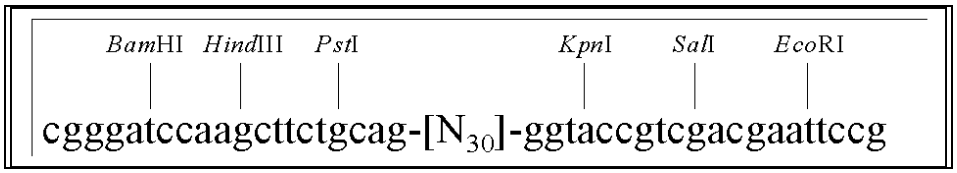

Fig. 16. Restriction sites within the 20 bp primers synthesized for use in the SELEX selection of DNAs containing SarA-binding sites.

DNA and maintain the complexity of the fragment pool, 40-50 µg of synthetic template will be used for a single round of primer extension using the complementary primer (primer 2). The resulting double-stranded DNA fragment will then be gel-purified and mixed with an appropriate concentration of SarA (the appropriate concentration of SarA will be determined empirically according to Pollock (32)). The bound DNAs will then be separated from the unbound DNAs using one of two methods. In the first, we will utilize our affinity-purified SarA antibody and a protein A-sepharose slurry to immunoprecipitate bound DNAs. After elution of bound DNAs from protein A-sepharose pellets, the DNA will be purified by extraction with phenol/chloroform and ethanol precipitation. The second is EMSA, in which case the bound-DNA fraction will be visualized by autoradiography and excised from the gel. The DNA's will then be purified by elution from the gel followed by phenol/chloroform extraction and ethanol precipitation. In either case, DNAs purified from the bound fraction will be subjected to a second amplification using both primer 1 and primer 2. To enhance the selectivity of the protocol, this cycle of binding, purification and amplification will be repeated approximately 5-7 times (see below). In all amplifications, the number of cycles will be kept relatively low (~14-20) to avoid depletion of the nucleotide pool and generation of artifactual PCR products. Additionally, even when we use the immunoprecipitation protocol to purify bound DNAs, we will do EMSA using both bound and unbound DNA fragments to ensure that 1) the size of the amplification product (as reflected by the size of the unbound fraction) is appropriate and 2) the size of the bound fraction is relatively constant (as expected based on the binding of the same protein to a 70 bp DNA fragment). The fraction of bound DNAs should increase with respect to the total DNA loaded on the EMSA gels until a steady state level is achieved. At that point, the DNAs will be cloned by blunt-end cloning into a *SmaI* site and by taking advantage of the restriction sites engineered into the 20 bp flanking regions (Fig. 16). The latter will be employed to facilitate the generation of multiple clones while the former will be used to minimize the bias associated with the removal of fragments that contain the restriction enzyme site. Approximately 100 - 150 of the cloned DNAs will be sequenced using an ABI377 automated DNA sequencer located in the Department of Microbiology and Immunology. DNA sequences will be aligned using the PILEUP program resident in the Genetics Computer Group's software package. A consensus sequence will be derived based on the relative abundance of each nucleotide at each position (Fig. 15).

Once we have derived the consensus sequence defining a SarA-binding site, we will test the specificity of our selection protocol by comparing the affinity of different DNAs for SarA. Specifically, the sequence alignment from the PILEUP analysis will be arranged in a gradient ranging from those sequences that are most homologous to the consensus sequence to those sequences that are least homologous to the consensus. To determine the contributions of specific base pairs in the selected DNA to SarA binding, we will choose representative DNAs that are highly homologous, moderately homologous and somewhat homologous to the consensus and then determine the affinity (equilibrium dissociation constants) for SarA-binding using quantitative EMSA. The test DNAs will include pairs of fragments that have differences in a single base pair that is implicated as important based on its abundance in the pool of selected DNAs. For example, if the third position in the 30-mer is a guanine in a high proportion (e.g. 80%) of selected DNAs, we will test the contribution of that guanine by synthesizing a corresponding fragment that differs only at the third position. For quantitative EMSA, a limiting amount of ^{32}P-labeled DNA (<10 pM) will be equilibrated with various concentrations of SarA and subjected to native gel electrophoresis. The gels will be fixed, dried and exposed to X-ray film. Laser densitometry will be used to quantitate the amount of unbound DNA and the concentration of bound DNA will then be calculated by subtracting the amount of unbound DNA from the total concentration of input DNA. Bound DNA will be plotted against the concentration of SarA protein and an apparent K_D value derived from the concentration of protein that results in 50% complex formation. This procedure was successfully used by the Co-I in studies of the

trp repressor (15) and the myogenic transcription factors MyoD-E12 and myogenin-E12 (14).

Methods #2: Identification of S. aureus genes cis to SarA-binding sites and confirmation of SarA-mediated regulatory control. The primary goal of Specific Aim #3 is to identify *S. aureus* genes under the regulatory control of SarA. From the experiments described above, we will obtain important information regarding the DNA sequence that constitutes a SarA-binding site. This information will be used to search the *S. aureus* genomic database. While the utility of this approach is currently limited by the availability of sequence data, it is anticipated that the results of ongoing efforts to sequence the entire *S. aureus* genome will be publicly available in the near future. As this information is made public, we will use the FASTA searching algorithm resident in the GCG package to search the sequence database for sites that closely match the consensus SarA-binding site. The search parameters will be made degenerate based on the results of the PCR-ABS experiments (i.e. we will include alternative bases at sites that show no clear base preference) and will include any architectural parameters that are found to contribute to binding affinity. An example of such an architectural parameter is the spacing of the heptanucleotide repeats upstream of the *agr* P_2 and P_3 promoters). Putative binding sites will be considered likely candidates for SarA-mediated regulatory control only if they are *cis* to known or reasonable promoters. The genes *cis* to these promoters will then be identified in order to directly test for SarA-mediated regulatory control as described below. Again, our ability to identify target genes will be greatly facilitated by the availability of sequence data derived from the *S. aureus* genome. To test for SarA-mediated regulation of the genes identified by homology searches, we will do Northern blots using total cellular RNA isolated from wild-type *S. aureus* strains and their corresponding *sar* mutants. Northern blots will be done using DNA probes generated by PCR. To ensure that any regulatory effect is detected, RNA for Northern blot analysis will be isolated at various stages of *in vitro* growth (e.g. exponential *and* post-exponential growth). All of the protocols required to carry out the Northern blot analysis of individual target genes have already been described in earlier sections of this proposal. Although not included as part of this proposal, it is anticipated that we will eventually undertake experiments aimed at mutagenesis of genes found to be under SarA-mediated regulatory control and evaluation of the mutants using various animal models of staphylococcal disease.

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RESOURCE SHARING

Data Sharing Plan

Sharing of data generated by this project is
an essential part of our proposed activities
and will be carried out in several different ways. We would wish to make our results available both to the community of scientists interested in [this disease] and the biology of [its causative agent] to avoid unintentional duplication of research. Conversely, we would welcome collaboration with others who could make use of the vaccine assessment protocols developed in [the project].

Our plan includes the following:

Presentations at national scientific meetings. From the projects, it is expected that approximately four presentations at national meetings would be appropriate. There is an annual [Disease] Study Group meeting, of which the PI is secretary. This one-day meeting of interested persons presents new information on a variety of topics related to [the disease]. It is expected that the investigators from this [project] will be active participants of this focused group.

Annual lectureship. A lectureship has brought to the University distinguished scientists and clinicians whose areas of expertise were relevant to those interested in [the disease]. Lecturers have been [list of names]. Visiting lecturers will be scheduled to interact with the investigators of the project as appropriate with their specific areas of expertise which will provide an opportunity for members to present their work to the visitor.

Newsletter. The [disease interest group] publishes a newsletter which currently has a circulation of [number]. The newsletter's intent is to disseminate new information regarding [the disease]. The activities and discoveries of [the project] will be allocated 20% of the newsletter's coverage.

Web site of the Interest Group. The [interest group] currently maintains a Web site where information [about the disease] is posted. Summaries of the scientific presentation from the [quarterly project] meetings will be posted on this Web site, written primarily for a general audience. [Link to Web site]

Annual [Disease] Awareness week. Beginning this fall during the week of [date], the [interest group] will be sponsoring a [Disease] Awareness week. As part of that program, there will be a research poster display with discussions. In future years, [the project investigators] will be active participants in this program.

SAGE Library Data. [This project] will generate data from several SAGE libraries. It is our explicit intention that these data will be placed in a readily accessible public database. All efforts will be made to rapidly release data through publication of results as quickly as it is possible to analyze the experiments. Data used in publications will be released in a timely manner. SAGE data will be made accessible through a public site that allows querying as has been set up for a similar project. This site can be accessed at [link to Web site].

Model Organism Sharing Plan

CHECKLIST

TYPE OF APPLICATION *(Check all that apply.)*

☒ NEW application. *(This application is being submitted to the PHS for the first time.)*

☐ REVISION of application number: _____
(This application replaces a prior unfunded version of a new, competing continuation, or supplemental application.)

☐ COMPETING CONTINUATION of grant number: _____
(This application is to extend a funded grant beyond its current project period.)

INVENTIONS AND PATENTS
(Competing continuation appl. and Phase II only)
☐ No ☐ Previously reported
☐ Yes. If "Yes," ☐ Not previously reported

☐ SUPPLEMENT to grant number: _____
(This application is for additional funds to supplement a currently funded grant.)

☐ CHANGE of principal investigator/program director.
Name of former principal investigator/program director: _____

☐ CHANGE of Grantee Institution. Name of former institution: _____

☐ FOREIGN application ☐ Domestic Grant with foreign involvement List Country(ies) Involved: _____

☐ SBIR Phase I ☐ SBIR Phase II: SBIR Phase I Grant No. _____ ☐ SBIR Fast Track

☐ STTR Phase I ☐ STTR Phase II: STTR Phase I Grant No. ☐ STTR Fast Track

1. PROGRAM INCOME ***(See instructions.)***
All applications must indicate whether program income is anticipated during the period(s) for which grant support is request. If program income is anticipated, use the format below to reflect the amount and source(s).

Budget Period	Anticipated Amount	Source(s)

2. ASSURANCES/CERTIFICATIONS ***(See instructions.)***
In signing the application Face Page, the authorized organizational representative agrees to comply with the following policies, assurances and/or certifications when applicable. Descriptions of individual assurances/certifications are provided in Part III. If unable to certify compliance, where applicable, provide an explanation and place it after this page.
•Human Subjects; •Research Using Human Embryonic Stem Cells• •Research on Transplantation of Human Fetal Tissue •Women and Minority Inclusion Policy •Inclusion of Children Policy• Vertebrate Animals• •Debarment and Suspension; •Drug- Free Workplace *(applicable to new [Type 1] or revised [Type 1] applications only)*; •Lobbying; •Non-Delinquency on Federal Debt; •Research Misconduct; •Civil Rights (Form HHS 441 or HHS 690); •Handicapped Individuals (Form HHS 641 or HHS 690); •Sex Discrimination (Form HHS 639-A or HHS 690); •Age Discrimination (Form HHS 680 or HHS 690); •Recombinant DNA Research, Including Human Gene Transfer Research; •Financial Conflict of Interest (except Phase I SBIR/STTR); •Smoke Free Workplace; •Prohibited Research; •Select Agents •STTR ONLY: Certification of Research Institution Participation.

3. FACILITIES AND ADMINSTRATIVE COSTS (F&A)/ INDIRECT COSTS. See specific instructions.

☒ DHHS Agreement dated: 07/03/1995 ☐ No Facilities And Administrative Costs Requested.

☐ DHHS Agreement being negotiated with _____ Regional Office.

☐ No DHHS Agreement, but rate established with _____ Date _____

CALCULATION* *(The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as confidential information.)*

a. Initial budget period:	Amount of base $	175,000	x Rate applied	47.00%	= F&A costs	$	82,250
b. 02 year	Amount of base $	175,000	x Rate applied	47.00%	= F&A costs	$	82,250
c. 03 year	Amount of base $	175,000	x Rate applied	47.00%	= F&A costs	$	82,250
d. 04 year	Amount of base $	175,000	x Rate applied	47.00%	= F&A costs	$	82,250
e. 05 year	Amount of base $	175,000	x Rate applied	47.00%	= F&A costs	$	82,250
					TOTAL F&A Costs	$	411,250

*Check appropriate box(es):

☐ Salary and wages base ☐ Modified total direct cost base ☐ Other base *(Explain)*

☐ Off-site, other special rate, or more than one rate involved *(Explain)*

Explanation *(Attach separate sheet, if necessary.):*

Principal Investigator/Program Director (Last, First, Middle): Smeltzer, Mark Stephen

Place this form at the end of the signed original copy of the application.
Do not duplicate.

PERSONAL DATA ON PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

The Public Health Service has a continuing commitment to monitor the operation of its review and award processes to detect—and deal appropriately with—any instances of real or apparent inequities with respect to age, sex, race, or ethnicity of the proposed principal investigator/program director.

To provide the PHS with the information it needs for this important task, complete the form below and attach it to the signed original of the application after the Checklist. **Do not attach copies of this form to the duplicated copies of the application.**

Upon receipt of the application by the PHS, this form will be separated from the application. This form will **not** be duplicated, and it will **not** be a part of the review process. Data will be confidential, and will be maintained in Privacy Act record system 09-25-0036, "Grants: IMPAC (Grant/Contract Information)." The PHS requests the last four digits of the Social Security Number for accurate identification, referral, and review of applications and for management of PHS grant programs. Although the provision of this portion of the Social Security Number is voluntary, providing this information may improve both the accuracy and speed of processing the application. Please be aware that no individual will be denied any right, benefit, or privilege provided by law because of refusal to disclose this section of the Social Security Number. The PHS requests the last four digits of the Social Security Number under Sections 301(a) and 487 of the PHS Acts as amended (42 U.S.C 241a and U.S.C. 288). All analyses conducted on the date of birth, gender, race and/or ethnic origin data will report aggregate statistical findings only and will not identify individuals. If you decline to provide this information, it will in no way affect consideration of your application. Your cooperation will be appreciated.

DATE OF BIRTH (*MM/DD/YY*)	SEX/GENDER
SOCIAL SECURITY NUMBER (last 4 digits only) XXX-XX-	☐ Female ☐ Male

ETHNICITY

1. Do you consider yourself to be Hispanic or Latino? (See definition below.) Select one.

Hispanic or Latino. A person of Mexican, Puerto Rican, Cuban, South or Central American, or other Spanish culture or origin, regardless of race. The term, "Spanish origin," can be used in addition to "Hispanic or Latino."

☐ **Hispanic or Latino**

☐ **Not Hispanic or Latino**

RACE

2. What race do you consider yourself to be? Select one or more of the following.

☐ ***American Indian or Alaska Native.*** A person having origins in any of the original peoples of North, Central, **or** South America, and who maintains tribal affiliation or community attachment.

☐ ***Asian.*** A person having origins in any of the original peoples of the Far East, Southeast Asia, or the Indian **subcontinent**, including, for example, Cambodia, China, India, Japan, Korea, Malaysia, Pakistan, the Philippine Islands, Thailand, and Vietnam. (Note: Individuals from the Philippine Islands have been recorded as Pacific Islanders in previous data collection strategies.)

☐ ***Black or African American.*** A person having origins in any of the black racial groups of Africa. Terms such as "Haitian" or "Negro" can be used in addition to "Black" or African American."

☐ ***Native Hawaiian or Other Pacific Islander.*** A person having origins in any of the original peoples of Hawaii, Guam, Samoa, or **other** Pacific Islands.

☐ ***White.*** A **person** having origins in any of the original peoples of Europe, the Middle East, or North Africa.

☒ Check here if you do not wish to provide some or all of the above information.