PI: **Muir, Tom**	Title: Peptide Autoinducers of Staphylococcal Pathogenicity	
Received: 07/05/2016	FOA: PA16-160	Council: 01/2017
Competition ID: FORMS-D	FOA Title: NIH Research Project Grant (Parent R01)	
2 R01 AI042783-16A1	Dual:	Accession Number: 3954056
IPF: 6661401	Organization: PRINCETON UNIVERSITY	
Former Number:	Department:	
IRG/SRG: SBCB	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 16: [redacted] Year 17: Year 18: Year 19: Year 20:	Animals: N Humans: N Clinical Trial: N Current HS Code: 10 HESC: N	New Investigator: N Early Stage Investigator: N
Senior/Key Personnel:	*Organization:*	*Role Category:*
Tom Muir	PRINCETON UNIVERSITY	PD/PI
Richard Novick	New York University School of Medicine	Co-Investigator

OMB Number: 4040-0001
Expiration Date: 06/30/2016

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE	**State Application Identifier**

1. TYPE OF SUBMISSION*

❍ Pre-application ● Application ❍ Changed/Corrected Application

4.a. Federal Identifier
AI042783

b. Agency Routing Number

2. DATE SUBMITTED | **Application Identifier**

c. Previous Grants.gov Tracking Number

5. APPLICANT INFORMATION **Organizational DUNS*:** [redacted]

Legal Name*: PRINCETON UNIVERSITY
Department:
Division:
Street1*: PRINCETON UNIVERSITY
Street2: [redacted]
City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted]
ZIP / Postal Code*: [redacted]

Person to be contacted on matters involving this application
Prefix: First Name*: Maureen Middle Name: Last Name*: Thompson-Siegel Suffix:
Position/Title: Sr Grant & Conract Admin
Street1*: [redacted]
Street2:
City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted]
ZIP / Postal Code*: [redacted]
Phone Number*: [redacted] Fax Number: Email: [redacted]

6. EMPLOYER IDENTIFICATION NUMBER *(EIN) or (TIN)** [redacted]

7. TYPE OF APPLICANT* O: Private Institution of Higher Education

Other (Specify):
Small Business Organization Type ❍ Women Owned ❍ Socially and Economically Disadvantaged

8. TYPE OF APPLICATION*

❍ New ● Resubmission
❍ Renewal ❍ Continuation ❍ Revision

If Revision, mark appropriate box(es).
❍ A. Increase Award ❍ B. Decrease Award ❍ C. Increase Duration
❍ D. Decrease Duration ❍ E. Other *(specify)* :

Is this application being submitted to other agencies?* ❍ Yes ● No What other Agencies?

9. NAME OF FEDERAL AGENCY*
National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER
TITLE:

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT*
Peptide Autoinducers of Staphylococcal Pathogenicity

12. PROPOSED PROJECT
Start Date* Ending Date*
04/01/2017 03/31/2022

13. CONGRESSIONAL DISTRICTS OF APPLICANT
NJ-012

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: First Name*: Tom Middle Name: Last Name*: Muir Suffix:
Position/Title: Professor
Organization Name*: PRINCETON UNIVERSITY
Department:
Division:
Street1*: [redacted]
Street2: [redacted]
City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted]
ZIP / Postal Code*: [redacted]
Phone Number*: [redacted] Fax Number: [redacted] Email*: [redacted]

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested* [redacted]
b. Total Non-Federal Funds* [redacted]
c. Total Federal & Non-Federal Funds* [redacted]
d. Estimated Program Income* [redacted]

16.IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?*

a. YES ❍ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ● PROGRAM IS NOT COVERED BY E.O. 12372; OR

❍ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

● I agree*

** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.*

18. SFLLL or OTHER EXPLANATORY DOCUMENTATION File Name:

19. AUTHORIZED REPRESENTATIVE

Prefix: First Name*: Jeffrey Middle Name: Last Name*: Friedland Suffix:
Position/Title*: Director
Organization Name*: The Trustees of Princeton University
Department: [redacted]
Division: [redacted]
Street1*: [redacted]
Street2: [redacted]
City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted]
ZIP / Postal Code*: [redacted]
Phone Number*: [redacted] Fax Number: Email*: [redacted]

Signature of Authorized Representative* [redacted]

Date Signed* [redacted]

20. PRE-APPLICATION File Name:

21. COVER LETTER ATTACHMENT File Name:Cover_letter-resubmission.pdf

OMB Number: 4040-0010
Expiration Date: 06/30/2016

Project/Performance Site Location(s)

Project/Performance Site Primary Location

❍ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: The Trustees of Princeton University
Duns Number: [redacted]
Street1*: [redacted]
Street2: [redacted]
City*: [redacted]
County: [redacted]
State*: [redacted]
Province:
Country*: [redacted]
Zip / Postal Code*: [redacted]
Project/Performance Site Congressional District*: NJ-012

Project/Performance Site Location 1

❍ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: New York University School of Medicine
DUNS Number: [redacted]
Street1*: [redacted]
Street2:
City*: [redacted]
County:
State*: [redacted]
Province:
Country*: [redacted]
Zip / Postal Code*: [redacted]
Project/Performance Site Congressional District*: NY-012

File Name

Additional Location(s)

Funding Opportunity Number: PA-16-160. Received Date: 2016-07-05T12:16:58.000-04:00

OMB Number: 4040-0001
Expiration Date: 06/30/2016

RESEARCH & RELATED Other Project Information

1. Are Human Subjects Involved?* ❍ **Yes** ● **No**

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ❍ Yes ❍ No

If YES, check appropriate exemption number: 1 __ 2 __ 3 __ 4 __ 5 __ 6 If

NO, is the IRB review Pending? ❍ **Yes** ❍ **No**

IRB Approval Date:

Human Subject Assurance Number

2. Are Vertebrate Animals Used?* ● **Yes** ❍ **No**

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ● Yes ❍ No

IACUC Approval Date:

Animal Welfare Assurance Number [redacted]

3. Is proprietary/privileged information included in the application?* ❍ **Yes** ● **No**

4.a. Does this project have an actual or potential impact - positive or negative - on the environment?* ❍ **Yes** ● **No**

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ❍ Yes ❍ No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place?* ❍ **Yes** ● **No**

5.a. If yes, please explain:

6. Does this project involve activities outside the United States or partnership with international collaborators?* ❍ **Yes** ● **No**

6.a. If yes, identify countries:

6.b. Optional Explanation:

	Filename
7. Project Summary/Abstract*	abstract-resubmission.pdf
8. Project Narrative*	Narrative-resubmission.pdf
9. Bibliography & References Cited	bibliography_Staph_resubmission.pdf
10.Facilities & Other Resources	Facilities-resubmission.pdf
11.Equipment	Equipment_Muir_resubmission.pdf

Funding Opportunity Number: PA-16-160. Received Date: 2016-07-05T12:16:58.000-04:00

A research program will be undertaken to study *agr* signal transduction in the commensal pathogen, *Staphylococcus aureus*. The accessory gene regulator (*agr*) locus found in all staphylococci encodes a quorum sensing (QS) circuit that controls the expression of virulence and other accessory genes. It consists of two oppositely oriented transcriptional units, of which one encodes four proteins, AgrBDCA, involved in production and sensing of an autoinducer peptide (AIP), and the other encodes a regulatory RNA that is the effector of target gene regulation. The finding that staphylococcal virulence can be inhibited through antagonism of this QS pathway has fueled tremendous interest in understanding the detailed mechanisms at play throughout the circuit. Building on recent breakthroughs that have allowed us to reconstitute much as the quorum sensing circuit using purified components, we propose to integrate chemical, biochemical, biophysical and genetic tools for the purpose of obtaining a deeper understanding into the molecular processes underlying the production and sensing of the autoinducer peptide (AIP) pheromone that is central to *agr* regulation. The program will move forward in three directions: **Aim 1**, identifying the key missing players in AIP biosynthesis; **Aim** 2, understanding how agonism and antagonism of the QS system relates to newly discovered conformational changes in the AIP receptor, AgrC, and; **Aim 3**, identifying novel modulators of *agr* through sophisticated target-based screens. These studies will lay the groundwork for the development of therapeutic strategies targeting *agr*, but also contribute to a fundamental understanding of QS systems of this type, which are pervasive in the low-GC bacterial phylum, Firmicutes.

Staphylococcus aureus (*S. aureus*) is an opportunistic pathogen capable of invading mucous membranes or soft tissue; once invasion occurs, the bacterium deploys a diverse arsenal of virulence factors to evade the host immune system and to facilitate spread of the infection in the host environment. A research program will be undertaken to study the central quorum sensing (QS) circuit, termed *agr*, which regulates the onset of virulence as a function of bacterial population size. Building on recent breakthroughs that have allowed us to reconstitute much of the circuit using purified components, we propose to integrate chemical, biochemical, biophysical and genetic tools to gain a deeper understanding into the molecular processes underlying *agr* regulation; these studies will provide fundamental insights into how a QS circuit such as *agr* operates at the molecular level and will lay the foundation for the development of new strategies for treating *Staphylococcus aureus* infections.

Laboratory:
The Department of Chemistry at Princeton University has recently moved into the Frick Laboratory, an entirely new Chemical Sciences facility, totaling 265,000 square feet. The building is located in the heart of the sciences neighborhood that connects disciplines such as genomics, neuroscience, physics, chemical and biological engineering, mathematics, and molecular biology. Three entire floors of the building are dedicated to new research space. Professor Muir and his lab group occupy laboratory, office and group conference space primarily on the third floor (with addition space for spectroscopy instrumentation in the basement of the building). The synthetic peptide and protein chemistries described in this proposal will be performed within his state-of-the-art laboratory, specifically designed for modern synthetic protein chemistry and extremely well equipped for the chemical synthesis or protein expression, purification and characterization of peptides and proteins and for small molecule synthetic organic chemistry (116 feet of hood space). The laboratory also has dedicated cold-room, tissue culture and dark rooms for biochemistry and cell biology studies.

Clinical: N/A

Animal: N/A

Computer:
Professor Muir and his lab group have multiple high-end computers for purposes of communications, data processing, access to the internet, calculations, and monitoring laboratory equipment. The University Office of Information Technology maintains the TIGRESS High Performance Computing Center to provide resources meeting the broad computational requirements of the University research community. Available software includes statistical and database management packages, as well as programs for computer analysis of nucleic acid and protein sequences.

Offices:
The new Chemical Sciences building has three office modules on each floor. Each faculty member is allotted one office and one adjacent meeting room. Each module also has a shared conference room.

Other:
Frick Laboratory is in close proximity to the structures housing the Department of Molecular Biology and the University's Lewis-Sigler Institute of Integrative Genomics promoting collaborations between researchers who have had many overlapping research interests and scientific interactions. Core facilities include; the genomics center, equipped with state-of-the art next generation DNA sequencing instruments including the Ion Torrent Sequencer needed for some of the proposed studies; a proteomics mass spectrometry center harboring a range of systems tuned to proteomics applications (additional Orbitrap instruments); a macromolecular crystallography facility which possesses state-of-the-art robotic screening infrastructure and X-ray sources; and the biological imaging center equipped with a number of state-of-the-art fluorescence microscopes for cell biological and organismal biology applications. Lewis Library is also in close proximity and houses the science and technology collections of the university. Princeton University supports a glass blower, mechanical workshop, on-site departmental computing support personnel, and an efficient administrative infrastructure which provides a wide variety of services including electronic online purchasing and facility maintenance.

Major Equipment:

The relocation of the Muir lab from Rockefeller to Princeton University provided an excellent opportunity to acquire new major equipment that will greatly facilitate our research. The new MicroTOF-Q II mass spectrometer (Bruker) will be an essential tool for the analysis of peptides/proteins synthesized and semi-synthesized in our lab. This mass spectrometer has high sensitivity and accuracy for identification of proteins and post-translational modifications. This instrument also allows for ms/ms analysis of peptides. Another new piece of equipment is the Liberty peptide synthesizer (CEM Corporation). This instrument is extensively used to synthesize peptides up to 40 amino acid in length for various applications in our chemical biology research. The Liberty synthesizer is particularly useful for the preparation of modified histone peptides that are the key players of this grant proposal. Analysis of the protein/peptide samples is facilitated by four analytical HPLC systems (Agilent) all with autosamplers and with an option of fluorescence signal detection with one of the instruments. Purification of peptides is facilitated by two dedicated preparative-scale HPLC systems (Waters) with peak-picking auto-purification capability. In addition to two standard AKTA FPLC systems (GE Healthcare) for protein purification, we have recently acquired a third system with a multiple angle light scattering detector (Wyatt Technology) that will greatly enhance our ability to characterize large protein complexes such as nucleosomes. Tissue culture work will be carried out in two biosafety cabinets (Baker) and mammalian and insect cells will be maintained in three CO_2 incubators. A Zeiss microscope has also been purchased, with fluorescence capabilities, to monitor cells during tissue culture. The Muir lab has a designated room for work with radioactive substances, equipped with a MicroBeta2 automated scintillation counter (Perkin Elmer). Other standard major lab equipment includes two Avanti J-26XP floor centrifuges and one Optima L-80 XP ultracentrifuge (Beckman), several imaging systems (Odyssey (Licor), ImageQuant (GE Healthcare)), two freeze dryers (Labconco, Millrock), incubators/shakers (ATR), fermentor (New Brunswick Scientific), a UV-Vis spectrophotometer (Agilent) and an Emulsiflex-C3 cell homogenizer (Avestin). We have also acquired a plate reader (Molecular Devices) to monitor absorbance, luminescence and/or fluorescence of multi-well plates, which will be crucial for several assays currently being developed in the lab. All of the above-mentioned instruments are located on the 3^{rd} floor of the Frick Chemistry Laboratory building. Muir lab initiated the development of the Protein Chemistry Center as a shared research facility on the lower level of the chemistry department. As core equipment for the Center we have acquired a BioCore 4000 system, Chirascan CD spectrometer, SX20 stopped-flow Reaction Analyser (Applied Photophysics) and Fluorolog3-11 fluorimeter (Horiba), which will be used at crucial stages of our projects to gain further insight into biophysical properties of our protein/peptide samples. We have also recently installed within the Protein Chemistry Center a proteomics workstation containing a variety of analysis software (SCAFFOLD, Proteome Discover, MASCOT) that will be used to analyze, in house, the various proteomics datasets acquired in the course of this research.

The Princeton University Screening Center (PUSC) is fully equipped to carry out all the designed experiments in the proposal. As for the compound collection, the Screening Center currently has 75,000 singleton compounds as 10 mM DMSO solutions. This collection is assembled to have maximum diversity with drug-like properties while being vastly differentiated from those compound collections at typical academic screening centers, such as the NIH MLPCN center. PUSC also has access to the 1.4 million Chiromics Maximum Coverage compound collection, which is custom built to be used in line with the ASMS screening modality. In support of all aspects of biochemical and/or cell-based functional assays, PUSC possesses fully upgraded Perkin-Elmer EnVision and Biotek Cytation 3 multi-mode plate readers. All small molecule dispensing is performed using the non-contact acoustic dispenser Labcyte ECHO 550 system which is capable of handling volumes as low as 2.5 nL with great accuracy. For bulk liquid and reagent handling in the preparation of the assay plates, PUSC uses the Agilent Bravo liquid handling system.

For affinity selection mass spectrometry (ASMS), PUSC has a custom designed ASMS system, which consists of the Dionex 2-dimensional SEC-reverse phase nano flow liquid chromatography system in line with the Thermo QExactive orbitrap mass spectrometer capable of reaching resolution of 140000. This instrument is capable of screening the Maximum coverage collection of 1.4 million compounds in a one week time frame. The data produced from these experiments will be analyzed using the CHALIS software also accessible through the PUSC. All data generated from the screens at the PUSC are further analyzed by using the Chemaxon InstantJChem cheminformatics platform in combination with Perkin-Elmer Spotfire for data visualization.

Other shared research facilities available for our research groups are also located on the lower level. The departmental NMR facility currently has seven spectrometers on site, 300-800 MHz, including three new Bruker Avance cryoprobe units assembled into a cluster. One of these units is 1H-optimized, one is 13C optimized and the third, a Cryo-QNP, has 1H, 13C, 31P and 15N capabilities. All three units have a fully robotic 120-sample carousel allowing them to run unattended 24 hours a day, seven days a week. Importantly, a new Bruker 800 MHz instrument with cryoprobe was installed January 2013. This instrument will be essential to some of the proposed NMR studies on PHP. The mass spectrometer facility has several instruments including new Agilent TOF, Q-TOF, GC-MS and HPLC triple quad spectrometers and a Thermo Orbitrap instrument. The TOF unit is configured for walk-up high- resolution molecular mass determinations. The Q-TOF has an integrated chip-based nanoflow HPLC for proteomics. The triple quad is optimized for quantitative metabolomic experiments, while the Orbitrap is configured for proteomic and metabolomics applications. The Chemistry Department retains a staff of five professionals to maintain and repair the shared instrumentation. These staff members collaborate with research groups on the optimal application of the equipment to a particular project, and instruct individual researchers on the use of the instruments.

OMB Number: 4040-0001
Expiration Date: 06/30/2016

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: | First Name*: Tom | Middle Name | Last Name*: Muir | Suffix:

Position/Title*: Professor
Organization Name*: PRINCETON UNIVERSITY
Department:
Division:
Street1*: [redacted]
Street2:
City*:
County:
State*: [redacted]
Province:
Country*:
Zip / Postal Code*:

Phone Number*: [redacted] | Fax Number: [redacted] | E-Mail*: [redacted]

Credential, e.g., agency login: [redacted]

Project Role*: PD/PI | Other Project Role Category:

Degree Type: | Degree Year:

	File Name
Attach Biographical Sketch*:	Muir_NIH_new_biosketch_June_2016.pdf
Attach Current & Pending Support:	

Tracking Number: GRANT12207716
Funding Opportunity Number: PA-16-160 . Received Date: 2016-07-05T12:16:58.000-04:00

PROFILE - Senior/Key Person

Prefix: First Name*: Richard Middle Name P. Last Name*: Novick Suffix:

Position/Title*: Professor of Microbiology and Medicine
Organization Name*: New York University School of Medicine
Department:
Division:
Street1*: [redacted]
Street2:
City*:
County:
State*: [redacted]
Province:
Country*: [redacted]
Zip / Postal Code*:

Phone Number*: [redacted] Fax Number: [redacted] E-Mail*: [redacted]

Credential, e.g., agency login: [redacted]

Project Role*: Co-Investigator Other Project Role Category:

Degree Type: MD,BA Degree Year:

File Name

Attach Biographical Sketch*: Novick.biosketch.6-14-16.pdf

Attach Current & Pending Support:

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Muir, Thomas W.	Professor of Chemistry
eRA COMMONS USER NAME (credential, e.g., agency login) [redacted]	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
University of Edinburgh, Edinburgh, UK	B.Sc. (1st Honors)	1985-1989	Chemistry
University of Edinburgh, Edinburgh, UK	Ph.D.	1989-1993	Organic Chemistry
The Scripps Research Institute, San Diego, CA	Postdoc	1993-1995	Bio-organic Chemistry

A. Personal Statement

In the Muir Lab, we have developed general protein engineering approaches that allow recombinant polypeptides and synthetic polypeptides (or other artificial molecules) to be ligated together through a normal peptide bond. This technology, which can be applied both in vitro and in vivo, opens up the world of proteins to the tools of organic chemistry by allowing the insertion of unnatural amino acids, posttranslational modifications and isotopic probes site-specifically anywhere into proteins. Our methods are now used by numerous laboratories worldwide, and have allowed a large number of questions to be addressed.

My lab provides a unique and exemplary training experience for students to pursue their research. We offer superb research facilities and equipment for the production of proteins and chromatin via protein semisynthesis. Other projects include exploration of the enzymology and mechanisms of inteins using semisynthetic and NMR approaches, understanding the molecular mechanisms including molecular recognition processes underlying the Agr quorum sensing circuit controlling virulence in *Staphylococci*, investigation of the role of histidine phosphorylation in eukaryotic cells, and the use of genetic and chemical biology methods to study and modify, for the purposes of protein engineering, intein protein splicing elements. Our lab offers an extremely collaborative spirit both within as well as with other research groups.

I have a strong history of mentoring young scientists and training graduate students and postdocs. The Muir lab has trained a large number of predoctoral and postdoctoral candidates, and all have gone on to successful independent careers. In total, there have been over 57 pre- and-postdoctoral candidates that have come through the Muir lab with many having assumed independent academic positions. To date, I have trained 26 PhD students (of whom 12 are women) and 31 postdoctoral fellows (of whom 11 are women). These individuals have obtained positions in industry and at leading academic institutions around the world (including UC Berkeley, UCLA, UCSF, University of Washington, EPFL - École Polytechnique Fédérale de Lausanne, Scripps Institute, Penn State, Texas A&M, University of Naples, Columbia Medical School, University of Queensland, and many more).

B. Positions and Honors

Positions and Employment

1993-1995 Postdoctoral Associate, The Scripps Research Institute, San Diego, CA
1995-1996 Senior Research Associate, The Scripps Research Institute, San Diego, CA
1996-2000 Assistant Professor and Head of Laboratory, The Rockefeller University, New York, NY
2000-2002 Associate Professor and Head of the Selma and Lawrence Ruben Laboratory, The Rockefeller University, New York, NY

2005-2011 Richard E. Salomon Family Professor and Head of the Selma and Lawrence Ruben Laboratory, The Rockefeller University, New York, NY
2005-2011 Director, Pels Family Center for Chemistry, Biochemistry and Structural Biology, The Rockefeller University, New York, NY
2011-present Van Zandt Williams Jr. Class of '65 Professor of Chemistry, Princeton University, Princeton, NJ
2015-present Chair of Department of Chemistry, Princeton University, Princeton, NJ

Honors and Awards
Pew Scholar in the Biomedical Sciences (The Rockefeller University)
Alfred P. Sloan Research Fellow (The Rockefeller University)
Burroughs-Wellcome Fund, New Investigator in the Toxicological Sciences (The Rockefeller University)
Irma T. Hirsch/Monique Weill-Caulier Trust Research Fellow (The Rockefeller University)
Leonidas Zervas Award from The European Peptide Society (The Rockefeller University)
Richard E. Salomon Family Professor (The Rockefeller University)
Kavli Fellow (U.S. National Academy of Sciences)
Fellow of the American Association for the Advancement of Science (AAAS)
Vicent du Vigneaud Award in Peptide Chemistry (American Peptide Society)
Irving Sigal Young Investigator Award (The Protein Society)
Distinguished Teaching Award (The Rockefeller University)
Winner of the New York Academy of Sciences Blavatnik Award for Young Scientists and Engineers (NYAS)
Jeremy Knowles Award (Royal Society of Chemistry)
Arthur C. Cope Scholar Award (American Chemical Society)
MERIT Award (US National Institutes of Health)
Fellow of The Royal Society of Edinburgh
Breslow Award in Biomimetic Chemistry (American Chemical Society)

C. Contributions to Science (from 150 peer-reviewed publications)

Specific highlights from our own work include:

***Structure and Function of Inteins*:** Protein splicing is a remarkable posttranslational process in which an intervening sequence, termed an intein, becomes excised from a host protein, the extein, in an autocatalytic manner. In protein trans-splicing the intein is split into two pieces and splicing only occurs upon reconstitution of these fragments. We have for many years studied the molecular details of protein splicing that occurs in cis and in trans. Indeed, through our efforts, and those of others, we now have a much clearer picture of the nature of catalysis for all the steps in the canonical protein splicing mechanism. In addition, new technologies have emerged from these basic mechanistic studies and these have been used to answer a number of biology questions.

- Muir TW, Sondhi D, Cole PA. Expressed protein ligation: a general method for protein engineering. Proc Natl Acad Sci U S A. 1998 Jun 9;95(12):6705-10. PubMed Central PMCID: PMC22605.
- Xu R, Ayers B, Cowburn D, Muir TW. Chemical ligation of folded recombinant proteins: segmental isotopic labeling of domains for NMR studies. Proc Natl Acad Sci U S A. 1999 Jan 19;96(2):388-93. PubMed Central PMCID: PMC15146.
- Shah NH, Dann GP, Vila-Perelló M, Liu Z, Muir TW. Ultrafast protein splicing is common among cyanobacterial split inteins: implications for protein engineering. J Am Chem Soc. 2012 Jul 18;134(28):11338-41. PubMed Central PMCID: PMC3535263.
- Liu Z, Frutos S, Bick MJ, Vila-Perelló M, Debelouchina GT, Darst SA, Muir TW. Structure of the branched intermediate in protein splicing. Proc Natl Acad Sci U S A. 2014 Jun 10;111(23):8422-7. PubMed Central PMCID: PMC4060664.

The regulation of Chromatin Structure and Function: We have developed a suite of chemistry-driven methods to study how post-translational modifications of the core histone proteins in chromatin regulate the

structure and function of the chromatin fiber. This has led to new insights into the flow and storage of epigenetic information in mammalian cells, information that has improved our understanding of the molecular basis of fundamental DNA-templated processes such as transcription and that suggests new routes for the treatment of human diseases, many of which have an epigenetic origin. Relevant papers are listed below.

- McGinty RK, Kim J, Chatterjee C, Roeder RG, Muir TW. Chemically ubiquitylated histone H2B stimulates hDot1L-mediated intranucleosomal methylation. Nature. 2008 Jun 5;453(7196):812-6. PubMed Central PMCID: PMC3774535.
- Fierz B, Chatterjee C, McGinty RK, Bar-Dagan M, Raleigh DP, Muir TW. Histone H2B ubiquitylation disrupts local and higher-order chromatin compaction. Nat Chem Biol. 2011 Feb;7(2):113-9. PubMed Central PMCID: PMC3078768.
- Lewis PW, Müller MM, Koletsky MS, Cordero F, Lin S, Banaszynski LA, Garcia BA, Muir TW, Becher OJ, Allis CD. Inhibition of PRC2 activity by a gain-of-function H3 mutation found in pediatric glioblastoma. Science. 2013 May 17;340(6134):857-61. PubMed Central PMCID: PMC3951439.
- Nguyen UT, Bittova L, Müller MM, Fierz B, David Y, Houck-Loomis B, Feng V, Dann GP, Muir TW. Accelerated chromatin biochemistry using DNA-barcoded nucleosome libraries. Nat Methods. 2014 Aug;11(8):834-40. PubMed Central PMCID: PMC4130351.

Protein Chemistry in Living Cells and Animals: We have for several years explored the possibility of performing protein chemistry inside living systems – in principle this would allow for protein structure and function to be controlled and manipulated in ways inaccessible to standard genetics. A number of technologies have emerged from this initiative – many of which have relied on insights emerging from our long-standing mechanistic studies of inteins, remarkable proteins which mediate protein splicing (a naturally occurring protein editing reaction). These include a variety of small molecule and optically controlled protein ligation reactions, which permit the spatial-temporal control of protein function in cells and living animals. Key papers are listed below.

- Mootz HD, Muir TW. Protein splicing triggered by a small molecule. J Am Chem Soc. 2002 Aug 7;124(31):9044-5. PubMed PMID: 12148996.
- Giriat I, Muir TW. Protein semi-synthesis in living cells. J Am Chem Soc. 2003 Jun 18;125(24):7180-1. PubMed PMID: 12797783.
- Schwartz EC, Saez L, Young MW, Muir TW. Post-translational enzyme activation in an animal via optimized conditional protein splicing. Nat Chem Biol. 2007 Jan;3(1):50-4. Epub 2006 Nov 26. PubMed PMID: 17128262.
- David Y, Vila-Perelló M, Verma S, Muir TW. Chemical tagging and customizing of cellular chromatin states using ultrafast trans-splicing inteins. Nat Chem. 2015 May;7(5):394-402. PubMed PMCID: PMC4617616.

Virulence Regulation in Staphyloccus aureus: In a separate area of work, we have worked for many years to understand the molecular details of virulence control in pathogenic *Staphylococci*. We have defined the molecular structure of a family of secreted peptides from *S. aureus* that control virulence in the organism through a conserved quorum sensing signaling pathway termed *agr*. *Agr* remains the best-characterized quorum sensing pathway in any Gram-positive organism and, given its biomedical importance, is now widely studied. Using a combination of chemistry, protein engineering and molecular genetics, we have figured out many aspects of the molecular mechanism of this critical process. This understanding has led to the rational design of global inhibitors of virulence in *S. aureus* that prevent infections in animal models and that thus have therapeutic potential. Key contributions are listed below.

- Mayville P, Ji G, Beavis R, Yang H, Goger M, Novick RP, Muir TW. Structure-activity analysis of synthetic autoinducing thiolactone peptides from Staphylococcus aureus responsible for virulence. Proc Natl Acad Sci U S A. 1999 Feb 16;96(4):1218-23. PubMed Central PMCID: PMC15443.
- Kee JM, Oslund RC, Perlman DH, Muir TW. A pan-specific antibody for direct detection of protein histidine phosphorylation. Nat Chem Biol. 2013 Jul;9(7):416-21. PubMed Central PMCID: PMC3686892.

- Wang B, Zhao A, Novick RP, Muir TW. Activation and inhibition of the receptor histidine kinase AgrC occurs through opposite helical transduction motions. Mol Cell. 2014 Mar 20;53(6):929-40. PubMed Central PMCID: PMC4004102.

Published work in my bibliography:
http://www.ncbi.nlm.nih.gov/sites/myncbi/tom.muir.1/bibliograpahy/40835818/public/?sort=date&direction=ascending

D. Research Support

Ongoing Research Support

NIH:NIGMS 5R37 GM086868 (PI: Muir) 8/1/03 – 7/31/17
"Structure, Function and Applications of Inteins"
The major goal of this project is to explore the enzymology and mechanisms of inteins using semisynthetic and NMR approaches.

NIH:NIGMS 5 R01 GM107047 (PI: Muir) 9/01/13 – 4/30/17
"Development and Applications of 'Designer Chromatin'"
The major goal of this project is to deepen our understanding of the molecular mechanisms underlying the regulation of chromatin structure and function.

NIH:NCI P01 CA196539 (PI: Allis, Co-PI: Muir) 7/01/15 – 6/30/20
"Oncohistones: Role of Histone H3 Mutations in the Oncogenesis of Pediatric Cancers"
The broad goal of this project is to develop a suite of chemistry-driven tools to study the detailed mechanism by which histone H3 mutations, oncohistones, associated with pediatric brain and bone cancers mis-regulate epigenetic control of gene expression, leading to disease.

[redacted]

Completed

NIH:NIGMS 5R01 GM095880 (PI: Muir) 12/1/10-11/30/14
"Chemistry and Biology of Protein Histidine Phosphorylation"
The major goal of this project is to investigate the role of histidine phosphorylation in eukaryotic cells.

NIH:NIAID 5R01 AI042783 (PI: Novick) 5/15/98 – 3/31/16
"Peptide Autoinducers of Staphylococcal Pathogenicity"
The major goal of this project is to understand the molecular mechanisms including molecular recognition processes underlying the Agr quorum sensing circuit controlling virulence in Staphylococci.
Role: Co-PI

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Richard P. Novick, M.D.

eRA COMMONS USER NAME (credential, e.g., agency login): [redacted]

POSITION TITLE: Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Yale University, New Haven, CT	B.A.	06/1954	Psychology
New York University School of Medicine	M.D.	06/1959	

A. Personal statement

As noted below, I discovered and characterized the agr system and its autoinducing thiolactone peptide ligands and have spent much of the past 25 years engaged in studying it. I have published some 45 research papers and 8 reviews/chapters on the system. Included in my studies were mouse experiments that demonstrated attenuation of murine staphylococcal infections by inhibitory variants of the AIP that blocked agr activation. To evaluate these infection studies, I developed luciferase reporter vectors that enabled in vivo monitoring of the infection by IVIS imaging. My studies were greatly aided by an exceptionally productive collaboration with Prof Tom Muir, formerly of Rockefeller University, now at Princeton. These studies have been continuously supported by NIH for the past 25 years. Our joint studies have by now proceeded to the point where the biochemical-biophysical aspects of the work are best handled by Professor Muir, and constitute aims 1 & 2 of the present application, whereas my lab is best suited to evaluating the efficacy, both in vitro and in vivo of agr-inhibiting compounds, and constitute aim 3.

B. Positions and honors

Positions and Employment

1961-1962	Postdoctoral Fellowship with Dr. M.R. Pollock, F.R.S. National Institute for Medical Research, London
1962-1963	Assistant Residency, Dept. of Medicine, Vanderbilt University Hospital, Nashville, TN. (Professor of Medicne, Dr. David E. Rogers)
1963-1965	Special Postdoctoral Fellowship with Professor R.D. Hotchkiss, The Rockefeller University, New York.
1970-1975	Research Associate Professor, School of Medicine, New York University.
1976-Present	Research Professor, Dept. of Microbiology, New York University, NY.
1969-1975	Adjunct Professor, Dept. of Microbiology, New York University, NY.
1981-1991	Director, The Public Health Research Insititute, New York, NY
1975-1993	Member, Chief, Dept. of Plasmid Biology, PHRI.
1993-Present	Investigator, Skirball Institute, NYUMC. Professor of Microbiology and Medicine, NYU School of Medicine.
2010-Present	Recanati Family Professor of Science, NYU School of Medicine

Honors

Phi Beta Kappa, Magna Cum Laude, Alpha Omega, M.D. with Honors, Borden Award,
Berson Alumni Achievement Award, Member, National Academy of Sciences, Master Researcher Award, NYUSOM, 2009, Recanati Family Professor of Science, 2010.

C. Contributions to science

1. **Discovery and characterization of plasmids in staphylococci and their carriage of staphylococcal β-lactamase and other resistances**
   By the early 1960's, β-lactamase-based penicillin resistance in Staphylococcus aureus had become a major clinical problem, addressed by the development of methicillin. In a study of methicillin resistance in β-lactamase-producing S. aureus, I observed that β-lactamase production could be lost, which led to the demonstration that it was plasmid-coded. This, the first demonstration of plasmids in S. aureus, led to my long-lasting interest in and study of plasmid biology. This work has had a major impact on clinical medicine, agriculture, and microbiology, as well as contributing importantly to the understanding of bacterial molecular genetics, and has led to the construction by my lab of a plasmid cloning vector system for S. aureus, now in worldwide use among staphylococcal researchers.
   a. Novick, R.P. and Richmond, M.H. (l965). Nature and interactions of the genetic elements governing penicillinase synthesis in Staphylococcus aureus. J. Bacteriol. 90, 467-480.
   b. Novick, R.P. and Schwesinger, M. (1976). Independence of plasmid incompatibility and replication control functions in Staphylococcus aureus. Nature 262, 623-626.
   c. Novick, R.P. and Hoppensteadt, F.C. (1978). On plasmid incompatibility. Plasmid 1, 421-434.
   d. Gruss, A., Ross, H.P., and Novick, R.P. (1987). Functional analysis of a palindromic sequence required for normal replication of several staphylococcal plasmids. Proc. Natl. Acad. Sci. USA. 84:2165-2169.

2. **Discovery of plasmid-determined heavy metal resistance**
   Given the well-known carriage and dissemination of multiple resistance genes by E. coli plasmids, Christine Roth, a technician and I screened the newly discovered 25 kb "penicillinase" plasmids for other resistances and found not only MLS resistance, but also resistance to mercury, cadmium, lead, arsenate, arsenite, and bismuth salts, present in various combinations. These studies led to extensive biochemical studies by others, notably Simon Silver, who identified the resistance mechanisms. They also demonstrated that bacteria were capable of developing resistance to a wide variety of environmental inhibitors in addition to antibiotics. And they impacted environmental microbiology significantly, as plasmid-carried and chromosomal metal resistances were soon found among a variety of microorganisms, especially in areas polluted by industrial wastes. Several of these resistance genes were found to be inducible, and their promoters have thus been useful additions to our vector system.
   a. Novick, R.P. and Roth, C. (l968). Plasmid-linked resistance to inorganic salts in Staphylococcus aureus. J. Bacteriol. 95, l335-l342
   b. Smith, K. and Novick, R.P. (l972). Genetic studies on plasmid-linked cadmium resistance in S. aureus. J. Bacteriol. ll2, 76l-772.
   c. Novick, R.P., Murphy, E., Gryczan, T.J., Baron, E. and Edelman, I. (1979) Penicillinase plasmids of Staphylococcus aureus: Restriction-deletion maps. Plasmid 2, 109-129

3. **Discovery that plasmid replication control requires that the plasmid replication initiator protein be destroyed after a single use so it cannot be re-utilized**
   Control of plasmid replication had been a topic of great interest ever since Jacob, Brenner and Cuzin had proposed that plasmids were attached to specific sites on the inner leaf of the cell membrane and were replicated passively as accessories to the cell's genome. Pritchard suggested instead that plasmids were autonomous and that their replication was regulated negatively, a suggestion that was confirmed by Nordstrom's isolation of plasmid mutants in E. coli with increased copy numbers. We found this also to be true for staphylococcal plasmids and found that in S. aureus, plasmid replication was controlled indirectly by regulation of the rate of synthesis of the plasmid replication initiator protein. However, if this protein were to accumulate in active form, it would obviate the control mechanism and so I proposed that the protein could be used only once and had to be inactivated thereafter. Avi Rasooly, a post-doc in the lab, confirmed this by demonstrating that the dimeric protein was inactivated at the end of the replication cycle by the attachment of a short oligonucleotide to the active site tyrosine of one of the protomers. This was a key result in plasmid biology as it confirmed my view, previously articulated in my 1980 Scientific American article, that the plasmid was not a simple genome accessory but was rather a self-regulating autonomous endosymbiont in its own right.

a. Rasooly, A. and Novick, R. (1993). Replication-specific inactivation of the pT181 plasmid initiator protein. Science 262:1048-1050.
b. Rasooly, A., Wang P-Z., & Novick, R. P. (1994). Replication-specific conversion of the Staphylococcus aureus pT181 initiator protein from an active homodimer to an inactive heterodimer. EMBO J., 13:5245-5251.
c. Rasooly, A., Wang P-Z., & Novick, R. P. (1994). Replication-specific conversion of the Staphylococcus aureus pT181 initiator protein from an active homodimer to an inactive heterodimer. EMBO J., 13:5245-5251.

4. Discovery of the agr system and its regulation by an RNA molecule and activation by thiolactone-containing peptides

Reports in the early 1980's of S. aureus mutants defective in the expression of several virulence factors suggested that the mutations might have affected a regulatory system and so I ran a Tn551 transposon screen and isolated an insertion in a gene, now known as agrA, which turned out to be the response regulator of a two-component signal transduction system (TCS), which I named the agr system. In a very productive collaboration with Prof. Tom Muir, we have studied this system at great length, discovering that it is activated by a unique thiolactone peptide that binds to the signal receptor, AgrC, and that the TCS activates transcription of a divergent promoter that determines the synthesis of a 517 nt regulatory RNA, RNAIII, that controls translation of many virulence genes. The system occurs as 4 allelic variants that, in heterologous combinations, inhibit activation of the TCS. We demonstrated that this inhibition could attenuate or block a staphylococcal subcutaneous abscess in mice and have analyzed this effect in some depth, most recently finding that the inhibitory peptide could be injected at a different site from the bacteria and was effective after a delay of up to 8 h.

a. Ji, G., Beavis, R., & Novick, RP (1995). Cell Density Control of Staphylococcal virulence mediated by an Octapeptide Pheromone. Proc. Natl. Acad. Sci. USA 92:12055 12059.
b. G., R. Beavis, and R. P. Novick. (1997). Bacterial interference caused by autoinducing peptide variants. Science. 276:2027-2030.
c. Wright JS 3rd, Jin R, Novick RP. (2005) Transient interference with staphylococcal quorum sensing blocks abscess formation. Proc Natl Acad Sci U S A. Feb 1;102(5):1691-6.

5. Discovery and in depth analysis of the SaPIs – highly mobile staphylococcal pathogenicity islands carrying and disseminating the genes for toxic shock toxin and other superantigens

Following the tampon-based outbreak of staphylococcal toxic shock in the late 1970's and the identification by Bergdoll and Schlievert of the responsible toxin, TSST-1, I was invited by Proctor & Gamble and by Johnson & Johnson to clone and characterize the toxin gene. Barry Kreiswirth, a student in the lab, cloned the TSS gene (tst) and we found that it was flanked by 15 kb of DNA that was absent from non-TSS strains. This 15 kb element turned out to be a highly mobile pathogenicity island that was induced by certain helper phages to excise and replicate and was packaged in small infectious particles composed of phage virion proteins. It is the only known source of TSST-1. This element, abbreviated SaPI1 (staphylococcal pathogenicity island 1), was the prototype of a very large family of similar elements, with most S. aureus strains containing one or more. We have since characterized these islands in great depth, aided by a very productive collaboration with Dr. José Penadés. It has become clear that these elements have a very important role in staphylococcal biology and pathobiology, contrbuting not only to horizontal gene transfer, but also to the well-being of the host organism, lagely by down-regulating the reproduction of infecting phages.

a. Lindsay, J. A., A. Ruzin, H. F. Ross, N. Kurepina, and R. P. Novick. (1998). The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in Staphylococcus aureus. Mol. Microbiol.. 29:527-543.
b. Ubeda C, Barry P, Penadés JR, Novick RP. A pathogenicity island replicon in Staphylococcus aureus replicates as an unstable plasmid. Proc Nat Acad Sci, US, 2007; 104: 14182-88.
c. Ram G, Chen J, Ross HF, Novick RP. Precisely modulated pathogenicity island interference with late phage gene transcription. Proc Natl Acad Sci U S A. 2014 Oct 7;111(40):14536-41
d. Chen J, Ram G, Penadés JR, Brown S, Novick RP. Pathogenicity island-directed transfer of unlinked chromosomal virulence genes. Mol Cell. 2015 Jan 8;57(1):138-49

Complete List of Published Work in MyBibliography:
http://www.ncbi.nlm.nih.gov/sites/myncbi/richard.novick.1/bibliography/40752281/public/?sort=date&direction=ascending

D. Research support

Ongoing Research Support

[redacted]

Completed research support

R01 AI22159 Novick (PI) 09/01/85 - 08/31/13
Molecular biology of TSST-1 and other superantigen toxins
This project is a study of the novel, mobile genetic elements encoding toxic shock toxin - and other superantigens.
Role: PI

Budget Justification – Muir Lab

PERSONNEL

Tom W. Muir (1 summer month) will serve as the principal investigator. In addition to guiding the project, he will be responsible for experimental design and for interpretation of all aspects of the synthetic peptide and protein chemistry, as well as structural and biochemical studies of agr proteins. Dr. Muir has considerable experience in the chemical synthesis and semisynthesis of proteins, and has worked on the study of agr quorum sensing for over 20 years.

Postdoc: Stephen Xie (12 months) is an experienced protein structural biologist with extensive training in the area of AgrC biochemistry. Dr. Xie joined the Muir lab to study the agr system and has spearheaded our efforts to study AgrC by x-ray crystallography. He will work on the experiments outlined in Aim 2.

Graduate Student : Aishan Zhou (12 months). Ms. Zhou is a third year graduate student in the Muir lab. She has received extensive training in the areas of peptide and protein chemistry, especially as applied to *agr*. Ms. Zhou has developed genetic assays and crosslinking approaches to study the AIP biosynthesis and AIP/AgrC binding. She will work on the biochemical and biophysical experiments outlined in Aim 1.

Graduate Student: to be appointed (12 months). He/She will work on the experiments outlined in Aim 3.

MATERIALS & SUPPLIES

I. HPLC/FPLC COLUMNS

There is an unusually large amount of analytical and semi-preparative reverse-phase HPLC/FPLC in the work proposed. Funds are requested for analytical and semi-preparative HPLC/FPLC columns. We have budgeted for:

- 3 analytical (4.6mm x 25cm, Vydac) columns at [redacted] each
- 3 semiPrep (1.0 x 25cm, Vydac) columns at [redacted] each
- 2 FPLC G75 size exclusion columns at [redacted] each (for AgrC purification work)

HPLC/FPLC Columns: [redacted]

II. SUPPLIES

The following supply costs are based on three full-time (one Postdoc and two Graduate Students) researchers working in the laboratory. Unless otherwise stated, all projected consumables have been calculated based on the actual current amounts used in peptide/protein chemistry in the Muir laboratory.

A. Chemicals

Misc. Organic Chemicals

Specialty chemicals are required on a continuing basis for the synthesis of modified amino acids containing stable isotopes and fluorescent probes, of dipeptide analogs, of unusual intermediates, and of starting resins and resin linkers. An amount of [redacted] per full time person per month, has been arrived at based on my own experience of the typical expenditures of a laboratory engaged chemical biology. This figure is in line with amounts used in synthetic organic research groups (adjusted for the estimated percentage time spent on organic non-peptide synthesis).
Misc. organic chemicals: [redacted]

B. Peptide Synthesis
We anticipate undertaking ~10 chemical syntheses of AgrD an peptides approximately 40 residues in length per year. All amounts are based on using the in-situ neutralization/HBTU activation method for Boc or Fmoc-based solid-phase peptide synthesis.

1). Boc-L-amino acids
Synthesis on a 0.05-0.5 Mmole scale requires 2.20 Mmole amino acid per residue which gives a total of 2.20x10-3 x 40 = 0.088 mole amino acids (of all kinds) per synthesis. At an average formula weight of 280 grams/mole for side-chain protected Boc-amino acids (from Nova Biochem), this equates to around 24 grams of high purity protected amino acid derivatives per total synthesis. For a synthesis of a 40 residue peptide at [redacted] per gram of protected amino acid x 24 g the cost is [redacted] per synthesis. We are budgeting for 30 syntheses per year.
Total Boc-L-amino acids: [redacted]

2). Solvents
We will require unusually large amounts of high purity solvents for stepwise solid phase peptide synthesis. Based on actual laboratory records we anticipate that the synthesis of each polypeptide will require the following:

- Dimethyl formamide (amine-free peptide synthesis grade), [redacted] per litre (Fisher) at 20 litres per synthesis amounting to 600 litres in year 1 [redacted]
- Dichloromethane (spectroanalysed grade), [redacted] per litre (Fisher) at 4 litres per synthesis amounting to 120 litres in year 1 [redacted]
- Trifluoroacetic acid (high purity synthesis grade), $150 per litre (Halochem) at 30 litres in year 1 [redacted]

Total solvent costs: [redacted]

3). SPPS Chemicals
A range of high quality reagents are required for the total synthesis of peptides, namely:

- High purity loaded PAM-resins (4g of each, Applied Biosystems), MBHA resin (20g, Peninsula Labs.), PEGA resin (50g,Novabiochem), Sulfonamide resin (Novabiochem), ninhydrin reagents, scavengers in year 1 [redacted]
- Diisopropylethylamine (Applied Biosystems), 600ml year 1
- HBTU/HATU in year 1

Total SPPS Chemicals: [redacted]

Overall Total for Peptide Synthesis: [redacted]

III. MOLECULAR BIOLOGY/PROTEIN EXPRESSION

A. Cloning

Restriction endonucleases, high fidelity thermostable DNA polymerases, DNA ligase, dNTP and PCR primers are required on a continuing basis for the construction of new expression plasmids, PCR amplification, and DNA sequencing.

Total Cloning: [redacted]

B. Microbiological media

We anticipate that over the year 100 L of LB media will be used to induce overexpression of recombinant proteins by addition of IPTG to the final concentration of 1 mM. Thus, at the current price of IPTG ([redacted]/5g, Sigma) we request [redacted] for IPTG, and [redacted] for media and antibiotics (from Fisher).

Total Microbiological Media: [redacted]

C. Chromatography

Our studies will require a range of affinity matrixes including, chitin beads from New England Biolabs, and Glutathione resin from Pharmacia, and Ni2+-NTA agarose (for affinity chromatography), and conventional sorbents for liquid chromatography (from Pharmacia and TosoHaas). Various ultrapure chemicals and disposable bottletop 0.2mM filters will be required for the preparation of buffers suitable for the gel-permeation, ion-exchange and HPLC studies to be performed on the FPLC/HPLC systems. Centrifugal concentrators, dialysis tubing will be required for pre-, inter-, and postcolumn treatments of protein samples.

- Acetonitrile (Spectranalyzed Grade, Fisher Scientific
- Affinity chromatography media
- Salts, buffers, filterware
- Microconcentrators, centifugal filters

[redacted]

Total Chromatography: [redacted]

D. Proteases

Our studies require the use of specific proteases (Factor Xa, thrombin) to remove N-terminal leader sequences form expressed proteins.

Total Proteases: [redacted]

E. Electrophoresis

Ultrapure acrylamide and agarose, urea and SDS for standard agarose-gels and SDS PAGE. The cost for these general electrophoresis supplies is estimated at [redacted] per year.

Total Electrophoresis: [redacted]

F. Sequencing and oligo synthesis

Total Sequencing: [redacted]

TOTAL MOLECULAR BIOLOGY COSTS YEAR 1= [redacted]

TOTAL MATERIALS & SUPPLIES COSTS YEAR 1 = [redacted]

TRAVEL

A. We request [redacted] per year for travel to domestic scientific meetings and synchrotron trips; 1 per postdoc/grad student and PI.

Total Travel: [redacted]

PUBLICATION COSTS

We request [redacted] per year to meet the costs of publications and reprints.

Total Publication Costs: [redacted]

TOTAL MAT. & SUPPLIES, TRAVEL, PUBLICATION YEAR 1= [redacted]

Budget justification

Personnel: Funds are requested for the salary and benefits of a postdoc (12 months calendar). The postdoc will be proficient in microbiological techniques and the handling of mice. First year: [redacted] in salary and fringe benefits.

Animals: Funds are requested for the purchase and maintenance of the mice that will be required for the proposed experiments. Yearly request: [redacted].

Supplies: Funds are requested for supplies at the rate of ~[redacted] per year per researcher – which is the usual rate for workers in the Novick lab. First year's request: [redacted].

Miscellaneous: Funds are also requested for travel to one domestic meeting per year. First year: [redacted].

PHS 398 Cover Page Supplement

OMB Number: 0925-0001

Expiration Date: 10/31/2018

1. Human Subjects Section

Clinical Trial? ❍ Yes ● No

*Agency-Defined Phase III Clinical Trial? ❍ Yes ❍ No

2. Vertebrate Animals Section

Are vertebrate animals euthanized? ● Yes ❍ No

If "Yes" to euthanasia

Is the method consistent with American Veterinary Medical Association (AVMA) guidelines?

● Yes ❍ No

If "No" to AVMA guidelines, describe method and proved scientific justification

3. *Program Income Section

*Is program income anticipated during the periods for which the grant support is requested?

❍ Yes ● No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

*Budget Period	*Anticipated Amount ($)	*Source(s)

PHS 398 Cover Page Supplement

4. Human Embryonic Stem Cells Section

*Does the proposed project involve human embryonic stem cells? ❍ Yes ● No

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://grants.nih.gov/stem_cells/registry/current.htm. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

❑ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

Cell Line(s) (Example: 0004):

5. Inventions and Patents Section (RENEWAL)

*Inventions and Patents: ❍ Yes ● No

If the answer is "Yes" then please answer the following:

*Previously Reported: ❍ Yes ❍ No

6. Change of Investigator / Change of Institution Section

☑ Change of Project Director / Principal Investigator

Name of former Project Director / Principal Investigator

Prefix:

*First Name: Richard

Middle Name:

*Last Name: Novick

Suffix:

☑ Change of Grantee Institution

*Name of former institution:

New York University School of Medicine

PHS 398 Research Plan

OMB Number: 0925-0001
Expiration Date: 10/31/2018

Introduction	
1. Introduction to Application (Resubmission and Revision)	Introduction-resubmission.pdf
Research Plan Section	
2. Specific Aims	Specific_Aims-resubmission.pdf
3. Research Strategy*	Research_Strategy-Final_resubmission.pdf
4. Progress Report Publication List	
Human Subjects Section	
5. Protection of Human Subjects	
6. Data Safety Monitoring Plan	
7. Inclusion of Women and Minorities	
8. Inclusion of Children	
Other Research Plan Section	
9. Vertebrate Animals	Vertebrate_animals_Novick.pdf
10. Select Agent Research	
11. Multiple PD/PI Leadership Plan	
12. Consortium/Contractual Arrangements	Signed.face.pdf
13. Letters of Support	Letters_of_Support.pdf
14. Resource Sharing Plan(s)	
15. Authentication of Key Biological and/or Chemical Resources	
Appendix	
16. Appendix	

Funding Opportunity Number: PA-16-160 . Received Date: 2016-07-05T12:16:58.000-04:00

I am grateful for the opportunity to present a revised version of our application "*Peptide Inducers of Staphylococcal Pathogenicity",* first reviewed in February 2016 at the SBCB study section (18 percentile, priority score 32). It seems that the reviewers found the topic of great biomedical importance and that the original proposal was rated very highly – as one of the reviewers put it "*In all respects, it has very few weaknesses".* The reviewers further note "*the proposed experiments are likely to have a high impact",* with the potential to provide fundamental insights that could lead to new therapeutic strategies. The reviewers stressed the strength of our research team, stating that "*the investigators are peerless",* and that our work "*has resulted in new mechanistic insights at virtually every step in the pathway.*" Indeed, the reviewers seemed particularly enthusiastic about the mechanistic and biophysical studies the form the core of the proposal (aims 1 and 2), noting the multidisciplinary and innovative approaches to be taken and the body of supporting preliminary data provided. Altogether, the consensus seems to have been that the proposal was very strong with only minor weaknesses, which we address below.

Perhaps the biggest critique of the grant relates to the proposed mouse infection experiments described in Aim 3. The reviewers unanimously characterized this line of investigation as being premature, going as far to say that it may be more useful to consider this an option for future experiments. Given this consensus view, we have completely reorganized Aim 3, replacing the mouse work (now viewed as more long term, as per the suggestion) with an entirely new sub-aim in which we will attempt to identify a global AIP activator of the *S. aureus agr* response employing a peptide screening technology newly developed in my group. Unlike global inhibitors of the *agr* response, which have been known for over a decade, a global activator has never been described. Such a compound would be of interest as a way of disrupting *S. aureus* biofilms (which occurs upon *agr* activation), with presumptive utility as a coating for medical devices/implants. We propose to develop such a molecule using a novel peptide array system.

Critique #1 pointed out the challenges associated with identifying AIP binding sites on ArgC by performing crosslinking studies. We certainly appreciate the technical challenges here, however, newly added preliminary work strongly argues for feasibility. Specifically, we have now successfully used photocrosslinking followed by mass spec mapping to localize the AIP binding site to one region of the receptor, namely residues 75-90 (see revised Figure 5). In the future we will narrow down the site using a combination of additional mapping with strategically placed CNBr cleavage sites, targeted mutagenesis and genetically incorporated crosslinkers. Note that, as per the suggestion, we did consider using electrophilic amino acids for cysteine based crosslinking – analogous to Lei Wang's work – however this approach is chemically incompatible with the AIP thiolactone. Irrespective, the proposed crosslinking workflow works and can allow mapping of the site as was originally hoped.

The Critique also points out that some of the "*more ambitious experiments lack preliminary data and may fail."* There is, of course, no guarantee that any ambitious experiment will work. Perhaps, a more relevant question to ask is whether the questions posed are worthy of the asking. On this level, there appears to be no pushback from the reviewers, rather it is a question of approach. Importantly, our newly added preliminary data strongly supports feasibility. For example, we have now determined the x-ray crystal structure, to 2.25 Å, of the complete cytoplasmic domain of AgrC (see revised Figure 6). This breakthrough underwrites a major component of specific aim 2. The reviewers also questioned our ability to identify the proteases responsible for degradation of $AgrD^{C}$ and processing of AgrD(1-32)-thiolactone. Our work has already shone light on this – the importance of $AgrD^{C}$ turnover in driving AIP biosynthesis came out of our published studies, and the likelihood that SpsB is not the only (even the physiologically relevant) protease for processing AgrD(1-32)-thiolactone is supported by our extensive preliminary data, expanded on in the revision. We apologize if we failed to convey our appreciation of the attendant technical challenges here, and regret if we gave the impression that a one-dimensional approach will be taken. In fact, we will take both a candidate and unbiased approach to this problem, employing various approaches (Aim 1). In terms of the former, we have now generated a series of *S. aureus* strains with candidate proteases deleted or mutated (e.g. $clpP^{S98A}$), with many others in the works. As for the biochemical approach, we have now shown that the AgrD(1-32)-thiolactone is efficiently processed by the cell supernatant fraction of *S. aureus* cells, i.e. the protease(s) is a soluble secreted factor rather than a membrane protein (see revised Figure 4). This breakthrough greatly simplifies the identification of the protease(s) using either 'classical' fractionation methods, or by including photo-crosslinkers in non-hydrolysable AgrD(1-32)-thiolactone analogs. We stress that our expensive preliminary data, long track record of delivering the goods on this system and multifaceted and innovative approach to this problem, all argue for a successful outcome.

Reviewer #1 states that the "*single-molecule experiments are under-developed.*" We acknowledge that our discussion of this experiment was a little on the brief side and devote more space to this in the revision. Lastly, reviewer #3 states that "*innovation of the proposed experiments is not clearly described*". I respectfully disagree. Innovation is pervasive in the proposal, whether it is the use of a plethora of cutting-edge (and bespoke) chemical biology methods, new methods to generate and screen novel compounds, and the unprecedented use of a completely reconstituted two competent circuit in our mechanistic studies. Moreover, the proposal is laden with new concepts and hypotheses. For example, the link between quorum sensing and protein turnover, the proposed role of metabolic state in regulating the quorum sensing circuit, and the role of opposing mechanical motions in regulating the kinase activity of AgrC. Overall, we have tried very hard to strike a balance between asking interesting and biomedically relevant questions and proposing a mixture of innovative, as well as tried and tested, approaches to answer these. I hope and believe that the changes we have made in the revision more clearly articulate this vision. Key changes to the text are underlined (with the exception of Aim 3 which has been substantially reworked).

A research program will be undertaken to determine the molecular mechanisms underlying *agr* signal transduction in the commensal pathogen, *Staphylococcus aureus*. The accessory gene regulator (*agr*) locus found in all staphylococci encodes a quorum sensing (QS) circuit that controls the expression of virulence and other accessory genes. It consists of two oppositely oriented transcriptional units, of which one encodes four proteins, AgrBDCA, involved in production and sensing of an autoinducer peptide (AIP), and the other encodes a regulatory RNA that is the effector of target gene regulation. The finding that staphylococcal virulence can be inhibited through antagonism of this QS pathway has fueled tremendous interest in understanding the molecular mechanisms at play throughout the circuit. Such knowledge is expected to aid in the development of therapeutic strategies targeting *agr*, but also contribute to a fundamental understanding of QS systems of this type, which are pervasive in the low-GC bacterial phylum, Firmicutes. Significant progress has been made during the past funding cycle in understanding the mechanisms underlying *agr* signaling (see Research Strategy). Using reconstituted biochemical systems of defined composition, we now have a much clearer picture of AIP biosynthesis and secretion, as well as the molecular motions attendant to signaling through the two-component system. Our studies reveal many unanticipated features within the *agr* circuit and have generated a series of key biochemical questions that will form the basis of future studies, as outlined below:

Specific Aim 1: To determine the mechanisms of AIP biosynthesis and secretion. AIPs feature a thiolactone macrocycle and are produced from precursor polypeptides, encoded by AgrD, through two proteolytic events with an intervening secretion step. Thiolactone formation is coupled to the first of these processing steps and involves the activity of the integral membrane protease, AgrB, which converts AgrD into AgrD(1-32)-thiolactone and a C-terminal proteolysis fragment, $AgrD^{C}$. Our studies show that efficient thiolactone production requires rapid degradation of $AgrD^{C}$. We will explore this newly uncovered link between QS and protein homeostasis in *S. aureus* cells. As part of this, biochemical and genetic tools will be used to test the hypothesis that $AgrD^{C}$ is degraded by dedicated AAA+ proteases such as ClpS providing the thermodynamic driving force for AIP production. Our preliminary studies also indicate that AgrB is not responsible for secretion of AgrD(1-32)-thiolactone out of cells. Thus, this key step in the biosynthesis remains enigmatic. We will take both an unbiased and candidate based strategy, employing various chemical biology and genetic approaches, to identify the secretion apparatus required. As part of this, we will test the hypothesis that the phenol-soluble modulin ABC transporter (Pmt) is responsible for AIP secretion. The final step in AIP biosynthesis involves proteolytic removal of the an N-terminal leader peptide from the newly secreted, AgrD(1-32)-thiolactone. While it has been suggested that the general housekeeping protease, SpsB, is responsible for this step, our preliminary data casts considerable doubt on this assignment. Thus, we will carry out functional proteomic studies geared towards identifying the necessary protease for each of the four *agr* specificity groups.

Specific Aim 2: To understand the mechanism of activation and inhibition of the *agr* two component system. AIPs are recognized by the membrane-bound receptor histidine kinase, AgrC. Some AIPs are agonists of this receptor, while other are antagonists. Using AgrC reconstituted into nanometer-scale lipid bilayer discs, we have shown that ligand-induced activation and inhibition of the receptor occurs through opposite helical twisting motions that result in rheostat-like control over kinase activity. This breakthrough sets the stage for the next phase of the program where we will use photo-crosslinking strategies to pinpoint the ligand-binding pocket in AgrC and how this site couples to mechanical transduction. We will also perform structural studies on the soluble intracellular domain of AgrC engineered to be trapped in either the active or inactive state. Also proposed are single molecule biophysical studies designed to test the hypothesis that the rheostat-like behavior of AgrC activity reflects a continuum of dynamic states within the intracellular domain. As part of this aim, we will also initiate structural studies on the sensor domain of AgrC bound to agonist/antagonist ligands. Finally, we will explore the hypothesis, generated by our preliminary results, that *agr* activation is regulated by the metabolic state of the cell leading to down-regulation in times of stress.

Specific Aim 3: To identify new pharmacological modulators of the *agr* system. In this aim, we will identify novel inhibitors or activators of *agr* by performing high-throughput screens against reconstituted AgrC using newly established chemistries and biochemical assays. These studies will serve as starting point for exploring the viability of targeting *agr* for biomedical applications, a long-term goal of this program.

Collectively, these investigations will provide fundamental insights into how a QS system such as *agr* operates at the molecular level and will lay the foundation for the development of new strategies for treating *S. aureus* infections.

SIGNIFICANCE: *Staphylococcus aureus* (*S. aureus*) is part of the commensal microbial flora of ~30% of the adult population. In spite of its normal, beneficial nature, *S. aureus* is an opportunistic pathogen capable of invading mucous membranes or soft tissue [1]. Once invasion occurs, *S. aureus* is a remarkable, dynamic pathogen that is known to cause both acute and chronic illnesses such as bacteremia, sepsis, endocarditis and toxic shock syndrome [1]. As a consequence, *S. aureus* is a major health threat worldwide. Although the immune system and treatment with antibiotics can clear *S. aureus* infections, there are several risk factors that include a weakened immune system, surgery, and/or implanted medical devices that can lead to fatal infections [2]. Notably, these risk factors often persist in a hospital environment, where virulent *S. aureus* strains can thrive (nosocomial infections) and infect vulnerable patients being treated for an unrelated problem [3]. Such infections have only become more lethal with the emergence of antibiotic resistant, highly virulent strains of *S. aureus* (e.g. MRSA and VRSA) that can strike down healthy individuals in addition to the elderly and very young [4]. As an illustration, in 2005, there were over 278,000 MRSA-related hospitalizations, and estimates place MRSA-related deaths of at least 18,000 per year in the United States, which is nearly as many deaths as AIDS, tuberculosis and viral hepatitis combined [5]. Public concern over these 'superbugs' has risen dramatically in recent years as reflected by wide-spread coverage in the popular media (for a good example, see http://www.pbs.org/wgbh/pages/frontline/hunting-the-nightmare-bacteria/) and certainly there is a general consensus among microbiologists, physicians and public health experts alike that new therapies, including entirely new classes of antibiotics, are desperately needed to address this problem.

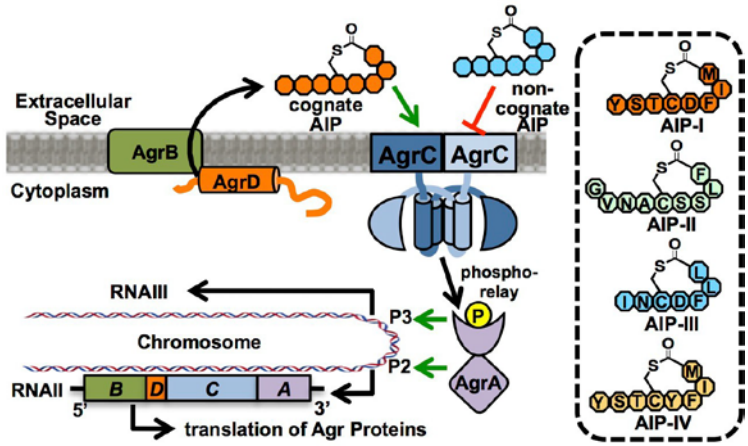

Figure 1: Virulence regulation in *S. aureus*. Schematic of the *agr* circuit. Activation of the *agr* response results from interaction of a cognate AIP-AgrC pair (i.e. within a specificity group) while non-cognate interactions are inhibitory. Inset: structure of AIPs I-IV.

S. aureus is an opportunistic pathogen that deploys a diverse arsenal of virulence factors to evade the host immune system and to facilitate spread of the infection in the correct host environment. The bacterium utilizes two main classes of virulence factors, each associated with different phases of population growth [6]. During the lag and early exponential phases, virulent *S. aureus* cells produce cell wall-associated factors that facilitate tissue attachment and evasion of the host immune system, allowing the bacteria to accumulate and possibly form a biofilm [7, 8]. For example, microbial surface components, recognizing adhesive matrix molecules, adhere to the extracellular matrix to give the bacteria an attachment point in the host. Protein A on the bacterial surface binds IgG antibodies to form a protective coat and evade the host immune system [7]. Once the bacterial population achieves a threshold density by late exponential phase, monitored by a quorum sensing (QS) system (see below), cell wall-associated factors are down-regulated, allowing for detachment from the original colonization site and establishment of an invasive infection [9]. At the same time, the bacterium secretes enzymes and toxins, termed exoproteins, to degrade host tissue and to promote spread of the infection. These include degradative enzymes such as proteases, hemolysins, as well as enterotoxins that are the causative agents of *S. aureus* food poisoning and contribute to toxic shock syndrome and other diseases by stimulating T-cells to produce proinflammatory cytokines in excess amounts [10]. Importantly, the coordinated expression of virulence factors is not only conserved within the Staphylococci [11], but also throughout the phylum *Firmicutes*, which includes pathogenic bacteria such as *Enterococcus faecalis* [12], *Listeria monocytogenes* [13], *Clostridium perfringens* [14], and *Clostridium botulinum* [15]. The onset of virulence in these organisms is regulated by a signaling network, including one QS system termed the accessory gene regulator (*agr*) [16].

Overview of the *agr* QS system: The *agr* locus consists of two oppositely oriented transcription units, of which one encodes four proteins, AgrBDCA, involved in producing or sensing of the autoinducer peptide, AIP, and the other encodes a regulatory RNA that is the effector of target gene regulation (Figure 1). *Agr* is universally conserved among the *Staphylococci* and its variation is thought to be the driving force behind speciation within the genus [11]. Indeed, there are 4 allelic variants of *agr* in *S. aureus* that differ in their ligand-receptor specificities, such that heterologous receptor-ligand interactions are in general inhibitory [17, 18]. All *S. aureus* AIPs contain a thiolactone macrocycle (Figure 1) and are produced through sequential processing of a ribosomally generated polypeptide encoded by the *agrD* gene product [19]. Sensing of AIP is achieved

through the AgrCA two-component system (TCS) in which AgrC is a membrane-bound receptor-histidine kinase (RHK) that undergoes autophosphorylation following cognate AIP binding and then transfers the phosphoryl group to a conserved response regulator (RR), AgrA. Phosphorylated AgrA dimerizes and binds to the genomic region between the *agr* promoters, activating both, thus completing a strong positive feedback loop [16, 20]. The P3 transcript encodes a regulatory RNA, RNAIII, that is the ultimate effector molecule of the *agr* response – at 514 nucleotides in length, RNAIII is a multifunctional molecule that acts as both a messenger (encoding δ-hemolysin) and an antisense RNA that regulates the expression of numerous genes [21-23]. Considerable effort has gone into defining the structure-activity relationships within the AIPs and AgrC that govern agonism and antagonism [19, 24-31]. These studies have mostly relied on cell-based assays involving coupled transcriptional readouts although, as discussed below, we have recently succeeded in reconstituting the TCS using purified components, thereby providing a platform for studying the detailed molecular mechanisms involved in signaling.

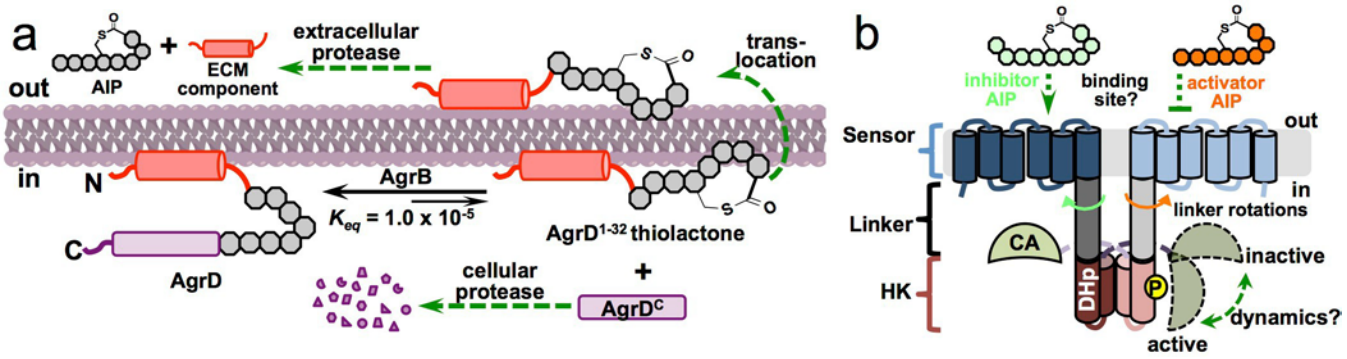

Figure 2: AIP biosynthesis and the *agr* TCS. (a) Schematics of AIP biosynthesis (**a**) and AgrC activation/inhibition (**b**). Outstanding questions are highlighted with green arrows. ECM, extracellular matrix.

AIP Biosynthesis: In the prevailing view of AIP biosynthesis (Figure 2A), the peptide is translated as a precursor, AgrD, in which the mature AIP sequence is embedded [24]. An N-terminal leader sequence forms an amphipathic helix that drives AgrD to the inner leaflet of the cell membrane where the first step in the maturation process occurs, namely processing of AgrD by a membrane-integrated cysteine peptidase, AgrB. Processing gives rise to two cleavage products, AgrD(1-32)-thiolactone and $AgrD^{C}$ [32, 33]. At face value, this step is expected to be thermodynamically unfavorable ($\otimes G^{o}$ = ~10 kcal/mol based on model reactions), due to the high energy nature of the thioester bond in AgrD(1-32)-thiolactone [34]. In a key breakthrough, we have recently shown that this process is driven by two critical features of the system: *(i)* membrane association of AgrD(1-32)-thiolactone, which stabilizes the macrocycle, and *(ii)* rapid degradation of $AgrD^{C}$, which affects the reaction equilibrium position, favoring product formation [34]. This work exposes a hitherto unappreciated connection between QS and protein homeostasis in *S. aureus* cells. The precise mechanism by which $AgrD^{C}$ is recognized and then degraded remains unknown, however our studies indicated that this must be an active process. Elucidation of this process will be a focus of Specific Aim 1.

The second step in AIP biosynthesis involves transport of the AgrD(1-32)-thiolactone intermediate to the outside of the cell where subsequent processing can then take place (Figure 2A). That translocation occurs before the second processing step is supported by the observation that large quantities of AgrD(1-32)-thiolactone can be detected in the extracellular matrix [35, 36]. AgrD lacks any signal peptide seen in proteins meant for secretion via the common Sec, Tat or Com secretion pathways [37, 38]. Consequently, it has been suggested that the AgrB protease is also responsible for the secretion step – i.e. processing and transport are somehow coupled [39]. However, AgrB (22 kDa) lacks any homology to known bacterial transporters (i.e. an ABC cassette) [40]. In preliminary data, we have engineered *S. aureus* cells to generate AgrD(1-32)-thiolactone independent of AgrB and used this system to show that AIPs are still efficiently generated and secreted even when *agrB* is genetically deleted (vide infra). This strongly suggests that AgrB is not the transporter for AgrD(1-32)-thiolactone. We imagine two possibilities; either AgrD(1-32)-thiolactone passively crosses the cell membrane, or another transporter system is involved. This key issue will be tackled in Aim 1.

The final step in AIP biosynthesis involves removal of the amphiphathic leader from AgrD(1-32)-thiolactone (Figure 2A). We have used a defined biochemical system to show that AgrB does not perform this step [34]. Indeed, the prevailing view in the literature is that this is carried out by the housekeeping serine protease, SpsB, which is attached to the outer leaflet of the bacterial membrane [41]. However, it is unclear how this single protease can correctly process the AgrD(1-32)-thiolactones from the four different *agr* groups in *S. aureus*, all of which have different sequences around the processing site (Figure 3C) . Moreover, AIPs from

groups I-IV all have different lengths, and there appears to be no room for sloppiness in the processing since addition or removal of residues from the mature AIP can convert an agonist into an antagonist of the response [19]. Indeed, in preliminary studies employing purified components, we have shown that processing of AgrD(1-32)-thiolactones by SpsB is extremely inefficient (see later). Based on this, we hypothesize that other (or additional) proteases are involved in this step and will conduct experiments to test this in Aim 1.

AgrC Activation: AgrC is a ~430-residue integral membrane protein that adopts a modular architecture shared among all members of the RHK family, with an N-terminal sensor domain, that spans the membrane 6 or 7 times, connected to a C-terminal histidine kinase domain that possesses all enzymatic activities required for the TCS and that is tightly regulated by AIP binding to the sensor domain [42-44]. Genetic and biochemical studies indicate that AgrC is an obligate dimer and that autophosphorylation between the protomer subunits can occur in trans [45]. We have succeeded in reconstituting purified recombinant AgrC-I (i.e. from group I *S. aureus*) into nanometer scale lipid bilayer discs (nanodiscs) [46]. This breakthrough has revealed several surprising features of the system. For example, we discovered the activity of AgrC is highly dependent on the presence of anionic lipids, which are present in *S. aureus* (and indeed most *Firmicutes*). We speculate that this represents an evolutionary adaption and creates a biochemical barrier to horizontal gene transfer into bacteria with lower levels of these lipids (i.e. Gram negatives where RHKs are absent). We also discovered that AgrC has a dramatically lower affinity for ATP (K_m = ~2 mM) compared other HKs (K_m typically in the 100s μM). We hypothesize that the atypically low affinity allows the *agr* response to be down-regulated in times of stress [47] – the cellular ATP concentration drops to sub-millimolar levels during stress or stationary phase [48]. Our bioinformatics analysis points to amino acid substitutions in the G1 box of the catalytic domain of AgrC as the origin of this low ATP affinity. Using this as a guide, we propose to engineer AgrC to confer higher ATP affinity on the receptor. By introducing this variant into *S. aureus* cells we will test, as part of Specific Aim 2, our hypothesis that the *agr* response is down-regulated by lowered ATP levels during stress.

Perhaps the central question for understanding *agr* regulation, and indeed any analogous QS circuit, is how ligand binding is transduced by the RHK into an intracellular output. For *agr* this is even more fascinating since, depending on the AIP, AgrC can either by activated or inhibited. Using our reconstituted system we have shown that the inhibitory interaction can either be neutral (i.e. does not affect the basal activity of AgrC) or involve inverse agonism (i.e. leads to a reduction of basal activity) [46]. Our studies also reveal the kinase and sensor domains in AgrC are connected by a helical linker whose conformational state exercises rheostat-like control over kinase activity (Figure 2B). Binding of AIP results in twisting of the linker in different directions (anti-clockwise in the case of agonism, clockwise in the case of inverse agonism). We hypothesize that this twisting motion alters the dynamic behavior within the kinase domain and will conduct a series of biophysical and structural studies to test this as part of Specific Aim 2.

Potential of *agr* as a Therapeutic Target: Interfering with the *agr* response has long been suggested as a route to combating *S. aureus* infections [25]. Since *agr* is not required for survival but for virulence, it has been argued that resistance development against *agr*-targeting reagents would be lower since they impose milder selection pressure versus classic, bactericidal or bacteristatic antibiotics [49]. AIP inhibitors have been shown to attenuate the spread of *S. aureus* infections in mice, phenocopying the use of agr-null *S. aureus* strains as infective agents [25]. Despite this result there has been considerable debate over whether inhibition or activation of *agr* would be the better option, with arguments in favor of the latter revolving around the ability to disperse persistent *S. aureus* biofilms through activation of the response [50]. At the very least, this strategy has potential in preventing the adhesion of *S. aureus* biofilms to medical implants. Regardless of which strategy, activation of inhibition, would be the more beneficial for treating an infection, a major limitation of all medicinal chemistry efforts in this area to date has been the reliance on peptide-based modulators, all of which have highly labile thiolactone linkages [51]. The instability of AIP peptides *in vivo* along with their immunogenicity make them poor candidates as drugs [52, 53], no doubt stymying progress in this area. In preliminary studies, we have exploited our ability to reconstitute AgrC in nanodiscs to perform high-throughput small molecule screens leading to the identification of several molecules that are competitive with AIPs. This work will form the basis of additional screening and chemistry efforts, as part of Specific Aim 3, designed to furnish the community with the first potent small molecule modulators of AgrC. In addition, we will develop a novel peptide array methodology in order to identify the first global activators of the *agr* response. Such agents will have utility both as research tools and as therapeutic leads.

INNOVATION: Uncovering the quorum-sensing dependence of exo-protein regulation represents a major advance in understanding *S. aureus* physiology and pathology [16]. The Muir/Novick team has made many of

the key contributions to our understanding of the *agr* response over the last 25+ years. Highlights include:

- Pioneering studies leading to the isolation, cloning and sequencing of the *agr* locus [21, 54, 55].
- Discovery of allelic variation within *agr* and subsequent characterization of bacterial interference between *S. aureus* specificity groups - studies that exposed the therapeutic potential of *agr* [17, 25, 47].
- Elucidation of the AIP structure and the development of the first synthetic routes to the molecule which, along which the establishment of robust cell-based assays, have allowed numerous structure-activity relationship studies to be performed on the system by many groups [19, 25, 26, 30, 31].
- The first demonstration that pharmacological inhibition of the *agr* response can attenuate the spread of a *S. aureus* infection in an animal [25]. Parenthetically, this work is, to our knowledge, the first example of targeting any QS system for therapeutic purposes.

While the pioneering studies summarized above have helped provide a roadmap for understanding the *agr* system [16], the reliance on cell-based transcriptional reporter assays has greatly hindered detailed mechanistic studies on the QS circuitry. With this in mind, we have over the last few years made a significant investment in establishing defined, biochemical systems for studying the biosynthesis of the AIP, as well as the engagement of this molecule with the AgrC receptor [46]. This successful reconstitution of an entire TCS module from highly purified components is without precedent and, as noted in the preceding section, has already answered a number of longstanding questions in the *agr* field, whilst at the same time revealing several hitherto unknown aspects of the system [46]. Likewise, reconstitution of the critical first step in AIP biosynthesis represents an important technical breakthrough that has provided quantitative insights into the molecular biosynthesis that would not have been possible otherwise [34]. In addition to these biochemical advances, we have also developed powerful chemical biology tools to study the *agr* circuit in *S. aureus* cells, including an orthogonal route to the AIP biosynthesis that allows us to uncouple AIP maturation from AIP secretion. Collectively, these new tools put our team in a unique position to tackle the various mechanistic questions outlined in this proposal. We anticipate this work will yield molecular insights into QS that will extend beyond the *S. aureus agr* system. Moreover, the proposed work is expected to lead to the identification of the first small molecule inhibitors of *S. aureus* virulence, as well as the first global activators of the *agr* QS system.

APPROACH: A research program will be undertaken to study the *agr* QS circuit responsible for virulence regulation in *S. aureus*. We will use chemical biology methods in conjunction with structural and genetic approaches to deduce the molecular mechanisms underlying the production and sensing of the AIP pheromone central to the QS circuit. These investigations will lay the foundation for the development of new strategies for treating *S. aureus* infections.

Aim 1. To determine the mechanisms of AIP biosynthesis and secretion.

AIP biosynthesis is a multistep process in which the AgrD precursor polypeptide is sequentially processed, leading to the secretion of the mature thiolactone-containing peptide into the extracellular milieu. Whilst there has been important progress in understanding AIP maturation, including our own work during the current funding cycle, several key aspects of the process remain mysterious. In particular, we know little of how AgrD(1-32)-thiolactone is secreted out of *S. aureus* cells and then further processed into the AIP, nor do we know the nature of the apparatus required for degradation of $AgrD^C$ which we have shown is intimately tied to the first step in the process. The goal of this aim is therefore to shed light on these remaining questions such that we might be able to reconstitute the entire AIP biosynthesis from purified components.

1.1: How is $AgrD^C$ rapidly degraded in *S. aureus* cells? The first step in AIP biosynthesis, involving AgrB-mediated processing of AgrD into AgrD(1-32)-thiolactone and $AgrD^C$, represents, to the best of our knowledge, the only known proteolytic event where an *S*-nucleophile serves as the final acceptor of the peptidyl group [34]. In principle, this step poses a significant thermodynamic challenge for the organism, given the high-energy nature of a thioester relative to an amide. Indeed, using a fully reconstituted system, we have shown that this step, which we refer to as proteolytic cyclization, is fully reversible and that the equilibrium lies far to the left (K_{eq} = 1 x10^{-5}). This finding raises the question of how enough AgrD(1-32)-thiolactone is produced *in vivo* to ultimately generate sufficient AIP to trigger the *agr* response. The concentration of AIP in culture medium reaches 5 μM within two hours of the onset of autoinduction [56]. Since every AIP produced also generates an equivalent of $AgrD^C$, simple calculations reveal that, all else being equal, the intracellular $AgrD^C$ concentration would be in low millimolar range [57]. This is hard to reconcile with the equilibrium behavior of proteolytic cyclization noted above - such a high $AgrD^C$ concentration would completely inhibit AIP production,

by lowering AgrD(1-32)-thiolactone levels. This analysis indicates that AgrD^{C} must be rapidly degraded in *S. aureus* cells (we estimate a half-life on the order of 10 s, [34]) in order that the reverse reaction be sufficiently suppressed to allow adequate flux of AgrD(1-32)-thiolactone through the pathway. We have experimentally validated this idea by over-expressing AgrD^{C} in *S. aureus* cells – as predicted this increases full-length AgrD levels whilst suppressing AIP production [34]. The focus of this sub-aim is to identify the proteolytic apparatus responsible for AgrD^{C} degradation. Both candidate-based and unbiased approaches will be taken, as follows.

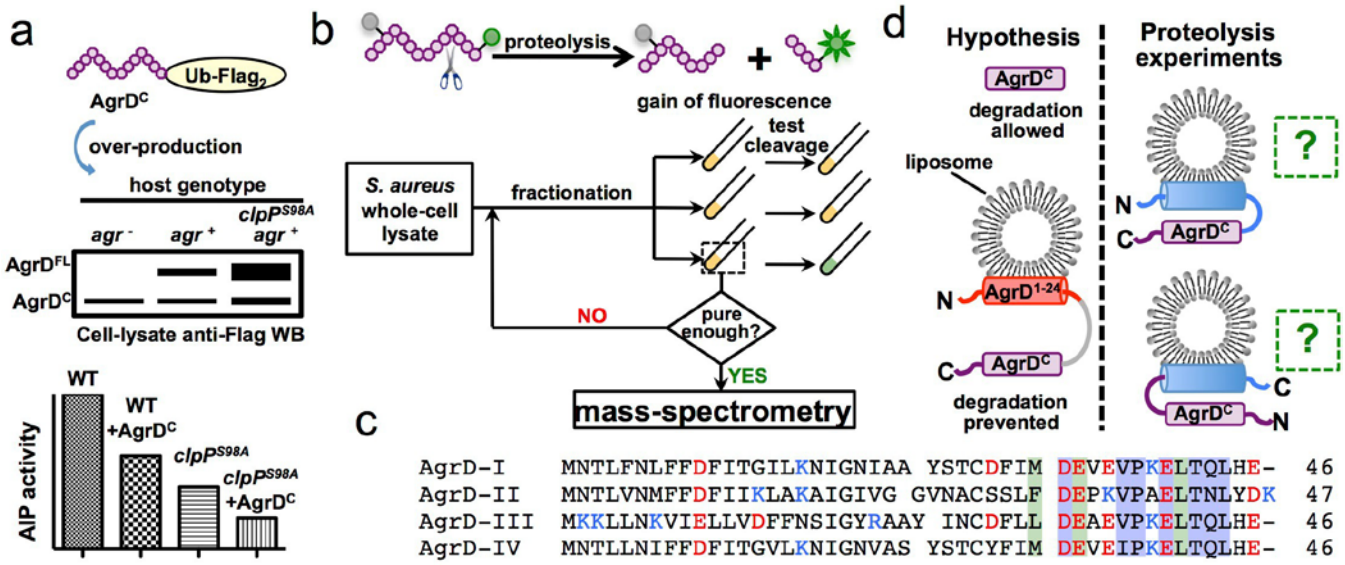

Figure 3: Identification of the proteolytic apparatus targeting AgrD^{C}. (a) Predicted effect of ClpP inactive mutation on AIP biosynthesis. (**b**). Identification of the AgrD^{C} protease using activity-guided fractionation employing a fluorescent substrate analog. (**c**) Alignment of *S. aureus* AgrD with a gap placed between AIP and flanking sequences. Residues involved in AgrB recognition and conserved residues without known function are highlighted in green and blue, respectively. (**d**) Does release of AgrD^{C} from the membrane, after proteolytic cyclization, trigger degradation? We hypothesize that artificially anchoring AgrD^{C} via an amphipathic helix (blue) to the membrane will prevent degradation.

There is considerable evidence that the ClpP-based AAA+ protease machinery (ClpCP or ClpXP) is required for virulence regulation in *S. aureus.* Both genetic deletion and chemical inhibition of ClpP, the catalytic subunit, lead to down regulation of the *agr* response [58, 59]. The molecular basis of this effect is unknown [60]. We hypothesize that this multimeric complex is directly responsible for degradation of AgrD^{C}. Several experiments are proposed to test this. Firstly, we will directly look at the levels of full-length AgrD, AgrD^{C} and AIP in an available ClpP-inactivated (*clpP^{S98A}*) *S. aureus* line [61] using a combination of immunoblotting (using tagged versions of AgrD and AgrD^{C}), mass spectrometry (for the AIP) and bioassays (for the AIP). We predict an increase in AgrD and AgrD^{C} levels, but a decrease in the amount of AIP (Figure 3A). Importantly, all of the tools required for this experiment are already available within the Muir laboratory [34]. We will also perform *in vitro* proteolysis assays using recombinant *S. aureus* ClpCP or ClpXP, generated using established methods [62, 63] and using synthetically generated AgrD^{C} from the four specificity groups as substrates. Importantly, the necessary materials for this work are also already in hand. We will employ an established RP-HPLC based assay to test whether the peptides are degraded in an ATP-dependent manner. While the AAA+ protease system is a strong candidate for the AgrD^{C} protease, we will nonetheless also take an unbiased approach to this problem. For this will we employ an activity-guided fractionation strategy to identify the protease responsible for degrading AgrD^{C}. Accordingly, we will use a fluorescence-based assay to follow the biochemical enrichment of protease activity in cellular lysates from *S. aureus* cells in late exponential phase (i.e. when *agr* is most active). We will follow standard multi-step fractionation schemes for identifying host enzymes. Key to this effort will be the development of an AgrD^{C} substrate peptide modified with a fluorescein-dabsyl donor-quencher FRET pair - degradation of the peptide will lead to a fluorescent signal (Figure 3B). Once the protease activity has been suitably enriched, we will use mass spectrometry to identify the proteins in the active fraction. Candidates will then be generated by recombinant expression in a suitable host and assayed for proteolytic activity as described above for ClpP-based AAA+ proteases. We will also validate the protease *in vivo* by making genetic deletions in *S. aureus* cells.

Once the protease that degrades AgrD^{C} is identified, experiments will be designed to pinpoint at AgrD^{C} sequence features recognized by the protease. Intriguingly, previous studies [64], as well as our own preliminary data, have shown that the most highly conserved residues within AgrD^{C} are not required for

substrate recognition by AgrB (Figure 3C). We speculate that these conserved motifs are recognized by the protease and will perform *in vitro* biochemical studies to test this, initially by performing an alanine-scan through the peptide sequence. Both the proteolysis activity by the wild-type protease and the physical interaction to a catalytically dead protease will be used as readouts. If key residues required for $AgrD^C$ degradation identified *in vitro* are not involved in AgrB recognition, we will transfect the corresponding mutant AgrD sequences into *S. aureus* and gauge the effect on proteolytic cyclization and on AIP production using established assays [34]. Alterations in the levels of starting material would provide strong *in vivo* evidence for a direct role of the protease in $AgrD^C$ degradation. In other studies, we will explore how the degradation machinery discriminates between full-length AgrD and $AgrD^C$ – the latter is a fragment of the former but is preferentially degraded in order for the *agr* circuit to function. We imagine two non-mutually exclusive possibilities. The first relates to the differential localization of full-length AgrD and $AgrD^C$ – the former is membrane associated whereas the latter is not [32]. Conceivable, this could affect substrate recognition by the protease for steric and/or electrostatic reasons. This will be tested by fusing an amphiphilic membrane localization sequence to either terminus of $AgrD^C$ and testing whether this remains a viable substrate for the degradative machinery using both cell-based and in vitro assays systems, the latter employing a liposome-based system (Figure 3D). Substrate specificity may also simply relate to peptide length, $AgrD^C$ is ~14 residues long *vs.* ~46 for full-length AgrD. This idea will be explored by systematically extending the length of $AgrD^C$ through the addition of extra residues to the N-terminus, in this case the in vitro assay will be used.

1.2 : How is AgrD(1-32)-thiolactone secreted from *S. aureus* cells? Following proteolytic cyclization, AgrD(1-32)-thiolactone is somehow transported to the outside of the cell, whereupon the final step in AIP biosynthesis can take place (Figure 2A). As noted earlier, AgrD lacks a canonical Sec, Tat or Com signal sequence, ruling out the involvement of these common secretion pathways in the transport step. In preliminary studies, we have explored whether AgrB is involved in AgrD(1-32)-thiolactone secretion, as some have suggested [39]. To this end, we have developed an intein-fusion strategy for producing AgrD(1-32)-thiolactone that is completely independent of AgrB (Figure 4A). This has allowed us to uncouple any role AgrB might have in transport from its known role in proteolytic cyclization. Remarkably, we find that AIP is still produced in the absence of AgrB, ruling out a role for this protease in the transport step. Given this result, we imagine two possibilities; either AgrD(1-32)-thiolactone can passively partition across the membrane, or an as yet unknown active transport system is involved. Both these possibilities will be explored. In the case of passive diffusion, we note that AgrD(1-32)-thiolactone is quite lipophilic ($LogP_{oct/water}$ = 0.6) - it can interact with the membrane both through the N-terminal amphipathic helical region [32] and through the conserved hydrophobic motif within the thiolactone macrocycle (Figure 3B) [34]. Thus, removal of the highly acidic $AgrD^C$ region during the first step might trigger an innate ability within the remainder of the AgrD sequence to partition across the membrane. To test this idea, we will exploit the reactivity of thioesters with nucleophiles such as aminooxy-containing reagents. Thus, we will generate liposomes containing an aminooxy-PEG, a reagent that cannot itself diffuse across membranes. These liposomes will then by incubated with a mixture of AgrD(1-32)-thiolactone and a highly charged peptide-thioester (poly-Glu), the latter serving as an internal control for assay integrity. Generation of a PEG adduct of AgrD, but not the control peptide, as gauged by SDS-PAGE and LCMS, would indicate that AgrD(1-32)-thiolactone can partition the membrane (Figure 4B).

Although a passive transport mechanism is worth testing, particularly given the lipophilicity of AgrD(1-32)-thiolactone, such a process would be unusual for a secreted signaling peptide [65]. Thus, the involvement of a hitherto unknown protein transporter system will also be explored. We will again take both candidate-based and unbiased approaches to this problem. Beginning with the former, we will test the hypothesis that the phenol soluble modulin ABC transporter (Pmt) is responsible for AgrD(1-32)-thiolactone secretion. Phenol soluble modulins (PSMs) are a class of secreted peptides produced by *S. aureus* during infections [66]. The surfactin-like properties of PSMs give them cytolytic activity towards a variety of cell types including neutrophils, red blood cells and even other bacteria [66]. Most PSMs are induced by the *agr* system; RNAIII actually encodes one PSM, namely δ-hemolysin, whereas the other two main classes of PSMs, PSMα and PSMβ, are directly regulated by phosphorylated AgrA [67]. Similar to AgrD, all PSMs contain an amphipathic helical region whose sequence varies considerably across the family (Figure 4C). All PSMs are actively secreted by the four-component Pmt complex [68], which we note is the only known transporter system up-regulated by *agr* [69]. Given this, along with the obvious similarity between PSMs and AgrD(1-32)-thiolactone (i.e. both are dominated by an amphipathic domain), we propose that Pmt is responsible for secretion of both. This will be tested by constructing an *S. aureus* strain lacking the genes for both Pmt and for PSMs – needed to ensure cell viability, as previously shown [68]. We expect this ⊗*pmt* strain to have a severely impaired ability

to generate AIPs as determined by bioassays and mass spectrometry analysis of supernatants using established protocols [30, 70]. We also propose an experiment designed to sterically trap an AgrD(1-32)/Pmt complex. In this case we will express in *S. aureus,* a AgrD(1-32) fused though its C-terminus to a bulky protein such as ubiquitin or GFP (Figure 4D). We expect this fusion peptide to recognized by Pmt via the amphipathic region, but then get stuck in the pore during transport, thereby providing us the opportunity to immunoprecipitate the complex. In a variation of this assay, we will make membrane preparations of *S. aureus* protoplasts using established methods [71] and incubate these with synthetic AgrD(1-32)-thiolactone peptides containing a diazirine photocrosslinker (photo-leucine) placed within the amphiphilic region (Figure 4D). Protocols for preparing and analyzing such peptides are well-established in the Muir group [72, 73]**.** Importantly, these assay systems can also be used in an unbiased manner, via proteomic analysis, to identify other putative transporters in the event that Pmt proves not to be the transporter.

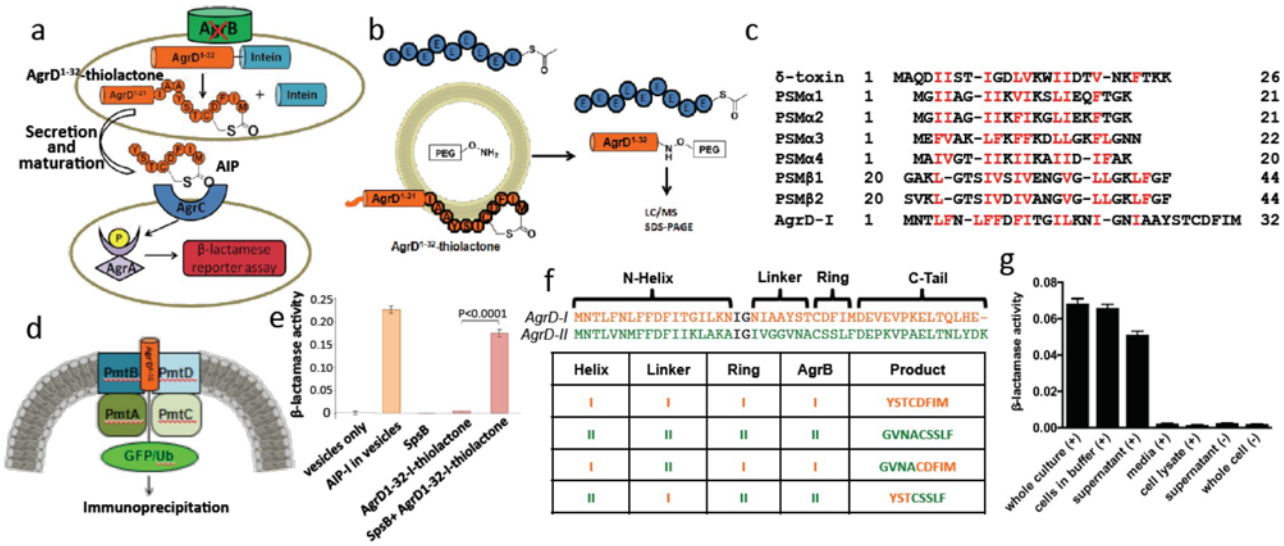

Figure 4: Studying the final steps in AIP biosynthesis. (a) Intein-fusion strategy used to show that AgrB is not involved in AgrD(1-32)-thiolactone transport. (**b**) Liposome-based assay to probe passive AgrD(1-32)-thiolactone membrane crossing. (**c**). Sequence alignment between PSMs and AgrDs. (**d**) Schematic of strategies proposed to trap a complex between AgrD(1-32)-thiolactone and a transporter protein, e.g. Pmt. (**e**) SpsB liposomes can process group I AgrD(1-32)-thiolactone to give active AIP as detected by a cell reporter bioassay. (**f**) Results of AgrD chimera experiments. (**g**) Addition of synthetic AgrDI(1-32)-thiolactone (+) to different cell fractions, followed by a bioassay for AIP production.

1.3 : How is AgrD(1-32)-thiolactone processed into the mature AIP? The four AIPs produced by the *S. aureus* specificity groups differ in both sequence and in length (Figure 3C). All are generated from the corresponding AgrD(1-32)-thiolactones through processing within a linker region that connects the amphipathic helix and the macrocycle. This linker is 8 residues long in all four groups and is of variable sequence - the exception is a conserved glycine residue at its N-terminus, which likely defines the end of the preceding amphipathic helix. What is remarkable is that the precise cleavage point within this linker varies from group to group (Figure 3C). Thus, the AIPs from groups I-IV all have different lengths. MS analyses of culture supernatants indicate that processing is precise in each group (data not shown). Moreover, we have previously shown that addition or removal of even a single residue from a native AIP can convert an agonist into an antagonist of the *agr* response [19]. To date, the only known protease implicated in the processing of AgrD(1-32)-thiolactone is SpsB, a type I signal peptidase that is attached to the outer leaflet of the bacterial membrane [41]. The catalytic domain of SpsB has been shown to cleave a short peptide corresponding to the linker region of a group I AgrD [41]. In preliminary results, we have extended this work by demonstrating that purified recombinant full-length SpsB embedded in a liposomal system can process the group I AgrD(1-32)-thiolactone to give detectable amounts of the native AIP (Figure 4E). However, the efficiency of cleavage is poor and, perhaps even more importantly, we have repeated this experiment with the group II AgrD(1-32)-thiolactone and in this case found no evidence of AIP production based on MS analysis or bioassays (data not shown). This result raises questions over whether SpsB is the only (or even the physiologically relevant) protease involved in the final step of AIP maturation. Indeed, it would be nothing short of remarkable if a single protease were capable of precisely processing all four groups, given the differences in AIP length. In support of this assertion, we have generated a series of *S. aureus* strains (based on an *agr* null background), each expressing a unique AgrD chimera (by swapping different regions from the 4 groups). The culture supernatants from these cells

were isolated and the AIPs generated characterized by mass spectrometry (a segment of this data is summarized in Figure 4F). This experiment reveals that the cleavage site (and hence length of the mature AIP) is defined by the sequence of the linker region. This strongly argues for the involvement of more than one protease in the final maturation step.

We will identify the protease(s) responsible for processing AgrD(1-32)-thiolactones using two orthogonal and unbiased approaches, namely (i) activity-guided fractionation employing synthetic AgrD(1-32)-thiolactone substrates and a combination of reporter cell assays and ms to identify active fractions, and (ii) affinity enrichment employing analogs of AgrD(1-32)-thiolactones containing non-hydrolysable amide isosteres (e.g. hydroxyethylene, phosphinates, reduced amides,) at the scissile peptide bond and if necessary photocrosslinkers to trap enzyme-substrate complexes for ms analysis. The extensive SAR analysis we have performed on the system (e.g. Figure 4F) will guide placement of these crosslinkers. In important preliminary studies, we have shown that the activity responsible for processing AgrDI(1-32)-thiolactone is present in the supernatant of *S. aureus* cells, i.e. it is a secreted soluble factor(s) rather than a membrane associated protein (Figure 4G). This breakthrough greatly simplifies the proposed biochemical studies, by allowing the use of standard chromatographic separations. Once candidate proteases are identified, we will verify using both biochemical methods, employing recombinant proteins, and using appropriate genetic deletions in *S. aureus.*

AIM 2. To understand the mechanism of activation and inhibition of the *agr* two component system. Perhaps the most remarkable aspect of the *agr* system is the bacterial interference phenomenon whereby AIPs from a different specificity group can actually inhibit the response [17]. This intra-group activation *vs.* inter-group inhibition operates through the receptor histidine kinase, AgrC. We have recently shown that AIP-induced agonism and antagonism of AgrC occurs through opposite helical twisting motions that result in rheostat-like control over autokinase activity [46] (Figure 2B). However, several key aspects of receptor regulation remain unresolved at the molecular level. The goal of this aim is to provide a detailed understanding of how ligand binding differentially affects signaling through changes in the structure and dynamics of AgrC.

2.1: Where is the AIP binding site on AgrC? While it has long been appreciated that AIPs bind the sensor domain of AgrC [27], the exact AIP binding site within this multi-pass membrane domain remains unknown. Without this information, it is difficult to formulate a testable hypothesis for how AIP binding leads to conformational changes in the helical linker that ultimately propagate to altered phosphohistidine levels. We will take a multi-pronged approach to this problem. Firstly, we will pursue a comprehensive photocrosslinking strategy to identify key contact residues within the sensor domain. In preliminary results, we have generated biotinylated analogs of AIP-I in which key residues within the macrocycle and the tail are replaced with diazirine or benzophenone containing amino acids (Figure 5A). These peptides not only retain the ability to activate AgrC nanodiscs, but become specifically crosslinked to the sensor domain following irradiation (Figure 5A). Using this *in vitro* system, we will map the AIP binding site by chemically digesting the crosslinked sensor domain followed by SDS-PAGE and MS analysis of the peptides, a process in which we have considerable experience [72, 73]. For this, we will use a CNBr digestion protocol employing engineered versions of the sensor domain with Met residues strategically incorporated in the sequence. Importantly, we have already generated several Met-engineered versions of AgrC-I (retaining full activity) for this purpose (data not shown). Note, the presence of the biotin handle on the AIP provides a useful enrichment handle for crosslinked peptides. Indeed, in new preliminary data, we have successfully used this crosslinking workflow to show that the macrocyclic region of AIP-I (employing M8pM-AIP-I, Figure 5A) interacts with a specific motif in AgrC-I encompassing residues 75-90 (Figure 5B). We will use this strategy to further refine the binding site of the thiolactone ring by moving the position of the crosslinker. By extension, by placing the crosslinker on the AIP tail, we expect to get additional information on the extent of the binding pocket in terms of primary sequence coverage. As a further extension of these studies, we will also incorporate a genetically encoded crosslinker, benzoylphenylalanine (BPA), into AgrC using amber suppression methods [74]. This powerful approach allows residue-level resolution from crosslinking studies without the need for MS analysis [75]. Importantly, in extensive preliminary studies we have shown that it is possible to generate active versions of AgrC-I nanodiscs containing site-specifically installed BPA (Figure 5C). Ultimately, we imagine that the bi-directionality of the two crosslinking strategies, i.e. from the ligand and from the receptor, will allow us to pinpoint the binding site of AIP-1 on its receptor, AgrC-I. The crosslinking studies will then be used to guide double mutant cycle experiments on the receptor and the ligand in order to confirm a direct interaction [76]. These studies will exploit our ability to determine the dissociation constant for the AIP-AgrC interaction using a fluorescence-based assay [46]. We will then go on to perform a similar series of studies to map the binding site of AIP-II on

AgrC-I. Notably, AIP-II acts as an inverse agonist of this receptor [77] and so it is conceivable that different residues on the receptor are involved in the interaction.

Currently, there is no high-resolution structural information available for the sensor domain of AgrC, or indeed any related sensor. Indeed, as note earlier, there is still some controversy over whether the sensor contains 6 or 7 transmembrane (TM) segments [42, 43]. Thus, depending on where the AIP binding site is mapped in the primary sequence from the above studies, it may be difficult to deduce how binding translates to mechanical motions in the linker helix region without some structural information on the sensor. An important exception to this situation would be if the AIP were found to engage residues in the last TM helix of the sensor. In this case, we would hypothesize that contacts would lead to conformational changes in the TM segment that would directly propagate into the linker helix given that we believe this to be one contiguous secondary structure element [46]. Unfortunately, the small size of the AgrC receptor (~45 kDa) places it well below the current size threshold for single particle cryo-EM studies on membrane protein preparations, in using nanodiscs [80-82]. Given this, we will attempt to identify an AgrC protein that is active and well behaved in a detergent-solubilized system suitable for crystallography – ligand affinity, autophosphorylation and monodispersity by SEC-MALS will be used to assess favorable properties. AgrC homologs from a number of Staphylococci species will be screened. In preliminary studies, we have already successfully over-expressed (in *E. coli*) and purified fully active AgrC homologs from all four *S. aureus* sub-groups as well as several other species including thermophiles (*Syntrophothermus lipocalidus*, data not shown and manuscript submitted). Importantly, these studies will encompass a series of constructs ranging from the full-length receptors down to the isolated sensor domains. Once a suitable candidate system is identified, we will initiate crystallization trials using dedicated sparse matrix screens optimized for membrane proteins (e.g. MembFac, MiTeGen etc). We do not underestimate the technical challenges associated with this undertaking (see Bio for previous structural work on membrane proteins in Muir lab). Indeed, this effort is likely to require multiple rounds of optimization, both at the level of construct engineering and crystallization conditions. Nonetheless, we are committed to this program and believe that the biomedical importance of AgrC coupled with the availability of a range of angonist and antagonist ligands make it a superior model system for understanding how ligand binding to an RHK sensor leads to transduction of a signal to the HK domain.

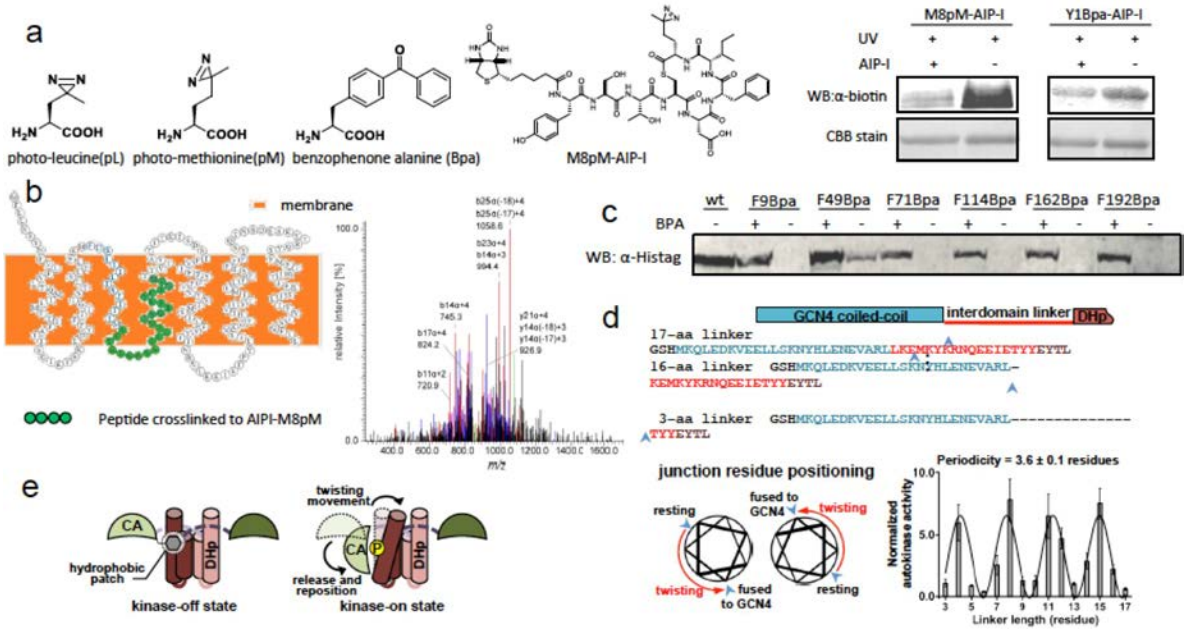

Figure 5: Understanding the mechanism of AgrC regulation. (a) Crosslinking AIP analogs to AgrC nanodiscs. **(b)** Results of crosslinking experiments using AIP-1 M8pM. Right: schematic of AgrC-I membrane topology with crosslink site shown in green. Left: example of ms data used to define the site of crosslinking. (**c**) Generation of AgrC nanodiscs containing Bpa using amber suppression methodology. (**d**) Use of GCN4-AgrC chimera proteins reveals helical twisting controls autokinase activity. (**e**) Model for AgrC activation based on unleashing of the CA domain from the bound state on the DHp domain.

2.2: How do ligand-induced helical-twisting motions in AgrC control kinase activity? Our assertion that twisting motions in the linker helix leads to changes in autokinase activity is based on two key experiments [46]. Firstly, we generated a series of soluble chimeric proteins (i.e. lacking the sensor domain) in which the leucine zipper protein, GCN4, was fused to the cytoplasmic domain of AgrC via the linker helix. By sequentially moving the fusion junction one residue at a time, we were able to turn the autokinase activity on and off in a periodic fashion in the absence of any AIP (Figure 5D). That the periodicity was exactly 3.6 ± 0.1

residues strongly argues for helical twisting. This remarkable result was then validated in the context for the full-length receptor by using a cysteine crosslinking strategy and employing a range of AIP ligands [46]. The assay not only confirmed that AIP binding induces twisting of the linker, but revealed this occurs in different directions from the basal state when an agonist binds compared to an inverse agonist (Figure 2B). This breakthrough raises the question of how exactly these twisting motions control autokinase activity. The linker helix connects the last TM segment of the sensor domain to the so-called *d*imerization and *h*istidine *p*hosphotransder (DHp) subdomain (Figure 2B), a helical hairpin region involved in homodimerization and containing the histidine phosphoacceptor residue. The DHp region is then connected via a flexible linker to the ATP-binding catalytic (CA) domain. We hypothesize that in the fully off state of the receptor, the CA domain engages an exposed surface on the DHp such that phosphorylation of the His is not permitted. In this model, binding of agonist leads to an occlusion of this DHp surface and hence unleashing of the CA domain, which is now able to engage the exposed histidine (Figure 5E). This type of repositioning has been proposed for other systems [83, 84]. We will employ structural and biophysical approaches to test this in the context of AgrC.

We propose to solve the x-ray structures of a series of GCN4-AgrC chimeras encompassing the "off-state", "basal-state" and "on-state" of activity. Such a structural series will provide numerous insights into the details of AgrC regulation. Note, the design of these proteins will be guided by our previous work, as discussed above [46]. In practice, two series of constructs will be used. The first series will encompass the entire cytoplasmic domain of AgrC (i.e. DHp + CA) fused to GCN4. In preliminary studies, we have succeeded in crystallizing the fusion construct corresponding to the "off-state". Using a combination of molecular replacement (using the structures of GCN4 coiled coil [85] and the isolated CA domain of AgrC [86] as search models) and SAD-phasing (employing selenomethionine containing constructs) we have now solved this structure at 2.25 Å resolution (Figure 6A). Consistent with our model (Figure 5E), this structure reveals that the CA domain is sequestered away from the active histidine through engagement with a surface at the base of the DHp domain (Figure 6A, inset). While we will continue to pursue structural studies of the other states, we are cognizant that the expected increase in dynamics (i.e. of the unleashed CA domain) associated with these constructs could make crystallization challenging. Thus, we will in parallel pursue structural studies on the corresponding GCN4-DHp constructs. These proteins are expected to be less dynamic, but should still provide insights into which surfaces within the DHp become exposed and occluded on going from the off- to on-state. Encouragingly, we have now obtained diffraction quality crystals of one of these GCN4-DHp constructs trapped in the 'on-state' (Figure 6B).

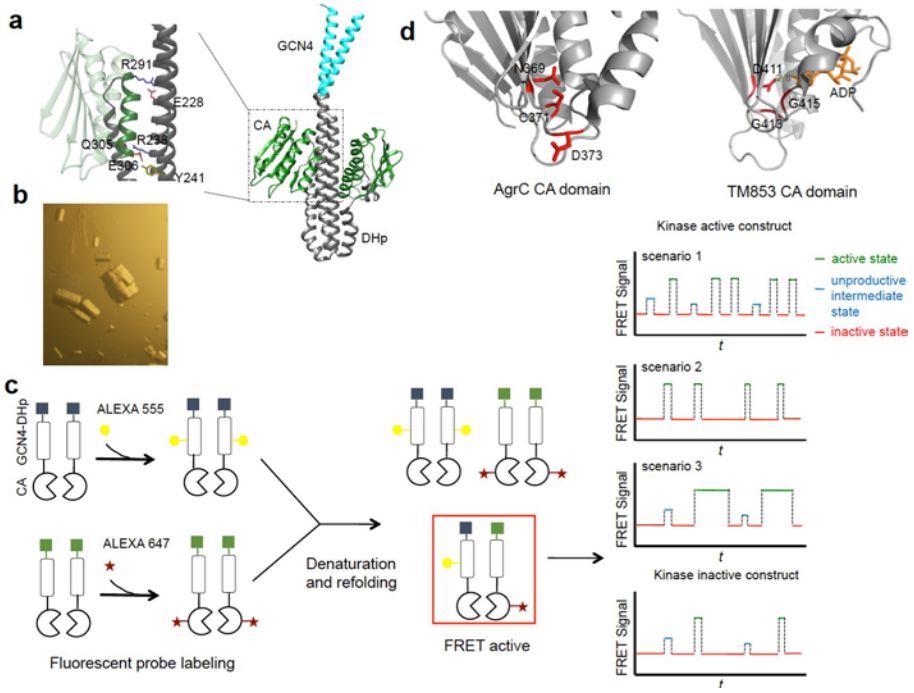

Figure 6: Effect of linker helix conformation on CA domain dynamics (a) Structure of AgrC-I cytoplasmic domain trapped in the 'off-state'. Inset: close-up of the DHp-CA interface. (**b**) Crystals of GCN4-DHp construct trapped in the active state. (**c**) Schematic of single-molecule experiment design to probe AgrC dynamics as a function of activity state. (**d**) Close-up of the CA of AgrC (pdb: 4BXI) and the CA of TM853, highlighting the difference in the G1 box residues.

In addition to high-resolution structural studies, we will also study the dynamic behavior of AgrC using single molecule fluorescence resonance energy transfer (FRET) experiments employing a total internal reflection (TIRF) microscope set-up (Figure 6B). These studies will be performed in collaboration Professor Haw Yang (Princeton University, letter attached), an expert in single molecule biophysics whose laboratory contains the necessary instrumentation for the proposed experiments. For convenience, we will employ our GCN4-AgrC chimeric constructs in these studies, again comparing the behavior of constructs that mimic a various activity states (Figure 5D). In practice, we will exploit the dimeric nature of these constructs and the fact that autophosphorylation occurs between protomers (i.e. in trans) within

AgrC [45]. Thus, an ALEXA 555 donor fluorophore will be incorporated into the DHp of one protomer using cysteine chemistry and an ALEXA 647 acceptor will be introduced into the CA domain of the other (Figure 6C). Several different pairwise combinations will be tested for an optimal FRET response – the newly available crystal structure of the AgrC cytoplasmic domain (see above) will also help guide the placement of fluorophores. These two species will then be mixed and subjected to a refolding protocol, thereby giving a statistical mixture, 50% of which will contain the desired donor-acceptor pair (note, the remaining components, containing one or the other probes, will be silent in the assay). In preliminary results, we have shown that the GCN4-AgrC chimeras can be denatured and then efficiently refolded into mono-disperse dimers with retention of full autokinase activity (data not shown). Our hypothesis predicts that constructs in different activity states will have different time-dependent FRET behavior (Figure 6c). Our recent structural data on the "off-state" construct (Figure 6A) suggests that it will primarily exhibit a single FRET state, with only occasional excursions to other states reflecting transient unleashing of the CA domain from its DHp docking site. By contrast, we predict the more active GCN4-AgrC constructs will exhibit a more complex FRET behavior reflecting disruption of this autoinhibitory CA-DHp interaction. This could be manifest in several ways including alteration in the dwell time in any given state (on or off) or a change in the number or distribution of FRET states observed (schematized in Figure 6c). A unique power of our system is the availability of GCN4-AgrC constructs spanning a range of activities. By comparing the single molecule FRET behavior of this activity series, it should be possible to rigorously model the physical characteristics of the active state of the kinase.

2.3: Is AgrC activity tuned to the metabolic state of *S. aureus* cells? In the final part of this aim, we will test our hypothesis that *agr* activation is internally regulated by the metabolic state of the cell, leading to down-regulation in times of stress. This idea derives from our discovery that the $K_{m\text{-}ATP}$ of AgrC is in a range (low millimolar) that makes it sensitive to changes in ATP/ADP levels that accompany stress [46]. Bioinformatic studies point to amino acid substitutions in the ATP-binding pocket of the CA domain as the origin of this low ATP affinity. Specifically, AgrC has a non-canonical G1 box compared to kinases that bind to ATP with higher affinity [46, 87-89]. Specifically, it lacks two conserved glycine residues and has an Asn residue in place of a conserved Asp residue (which engages the adenine ring of ATP) (Figure 6D). Using this information, we propose to engineer the CA domain of AgrC to have a higher ATP affinity. We will systematically mutate the G1 box, and if necessary second-shell residues, in AgrC-I with the view to lowering $K_{m\text{-}ATP}$ of AgrC into a range that would make the kinase less sensitive to falling ATP levels during times of stress. If necessary, we will employ the ROSETTA design suite developed by Baker and coworkers in these studies [90]. Our existing AgrC-nanodisc system will serve as the workhorse for these protein-engineering studies. Once a suitably engineered version of AgrC-I is developed, this will then be introduced into an appropriate *S. aureus* genetic background using established methods. Our hypothesis predicts that cells containing an altered *agr* circuit based on this engineered AgrC protein will have a dramatically different behavior during late-exponential and stationary phase, namely the *agr* response will not be down-regulated as in wild-type cells.

AIM 3. To identify new pharmacological modulators of the *agr* system (Completely NEW)

In this final aim, we will develop new chemistries, and exploit the availability of the reconstituted AgrC/AgrA assay system, to identify new chemical probes of *agr* that will lay the foundation for new therapeutic strategies.

3.2: Identification of a global activator of *agr*. Unlike global inhibitors of the *agr* response, which have been known for some time, a global activator (i.e. one that activates all four groups) has never been described. Such a compound would disrupt *S. aureus* biofilms (which occurs upon *agr* activation [9]), with presumptive utility as a coating for medical devices/implants. We propose to identify such a molecule using a peptide array system (Figure 7A). In preliminary studies, we have shown that AIP-I can be immobilized to a surface through its N-terminus via click chemistry. Importantly, the immobilized pheromone retains the ability to active the *agr* response in *S. aureus* reporter cells (Figure 7A, inset). With this as a launch pad, we will generate spatially defined arrays of AIP analogs using a novel chain inversion strategy. Briefly, we will employ standard SPOT peptide synthesis on a nitrocellulose surface functionalized with a bifunctional linker possessing an aryl hydrazine and an azide. A spatially defined library of AIP analogs will be synthesized off the hydrazine handle using standard Fmoc-SPPS and each peptide will be outfitted with an N-terminal PEG spacer followed by a propargyl group. Click chemistry will then be performed (thereby anchoring the peptide to the surface through both its termini) followed by protecting group removal and C-terminal cleavage. Subsequent oxidation of the C-terminal hydrazide (and spontaneous thiolactone formation [31]) will directly afford the immobilized library of thiolactone-containing AIPs. This strategy will allow us to quickly generate hundreds of AIP analogs, allowing unprecedented chemical diversity to be explored. As a convenient high-throughput readout of activity, we will employ our AgrC nanodiscs in conjunction with our available anti-pHis antibodies – activation of AgrC by an immobilized peptide will lead to autophosphorylation which can be readout in an

ELISA format. Hits will then be resynthesized and activity verified using established *in vitro* and cell-based assays. Identification of a global activator will open up many exciting new possibilities for exploring *agr* biology (including with our ongoing mechanistic work) and for future biomedical applications.

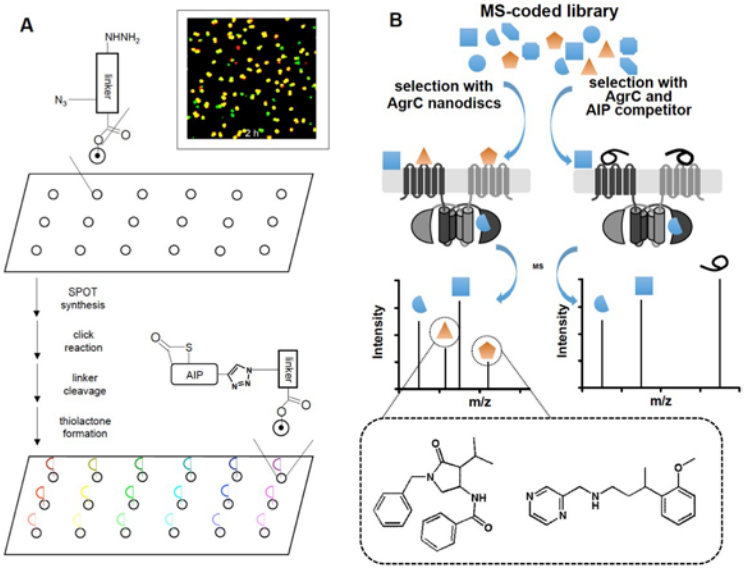

Figure 7: Identification of small molecule modulators of AgrC. (a) Proposed peptide array technology used to identify global *agr* activators. Inset: AIP-I immobilized through click chemistry to a passivated glass surface can still activate *agr* in *S. aureus* reporter cells (GFP readout). (**b**) Schematic of primary screen based on ASMS. SEC: size-exclusion chromatography. Bottom: hit molecules identified in proof-of-principle screen.

3.2: Identification of small molecule modulators or *agr*. The availability of AgrC-nanodiscs provides a powerful system for small molecule screens. In proof-of-principle studies, we have successfully applied an affinity-based small molecule screening (ASMS) strategy to discover AIP competitive binders of AgrC-I. These studies were performed in the Princeton Small Molecule Screening Center. The Center possesses both propriety (one million compounds) and commercial (~100,000 compounds) compound collections and is fully equipped to perform both phenotypic (i.e. cell-based) and targeted (i.e. using purified components) screens. In ASMS, a series of pooled compound mixtures (grouped based on mass considerations) are mixed with the target of interest, in this case AgrC-I-nanodiscs, and then resolved by size exclusion chromatography (SEC) [91]. Target-associated compounds are then identified by LC-MS/MS using a UPLC-Orbitrap system. In a pilot study, we have performed this analysis on ~50,000 compounds in the presence and absence of saturating amount of AIP-I. This has led to the identification of several AIP-competitive binders of AgrC-I (Figure 7B). Encouraged by these results, we will repeat the ASMS screen using the full compound collection available in the Center (see letter of collaboration from Center Director, Han Kim). Validation of the hits from the screen will involve multiple phases. Firstly, we will employ a newly developed high-throughput fluorescence-based assay involving displacement of a solvatochromic 4-dimethylamino-phthalimide labeled AIP from AgrC-nanodiscs [92]. We have shown that this system allows quantitative measurement of specific binding of ligands to the AgrC sensor domain (data not shown), making it an ideal secondary screening assay to measure sensor affinity of initial hits from SEC-based ASMS. Compounds that advance from this stage will then be assayed for their ability to activate or inhibit AgrC autokinase activity using our established biochemical [31, 46, 93, 94] and cell-based assays [95]. These lead molecules will serve as starting points for medicinal chemistry efforts designed to improve both potency and efficacy, as monitored by the in vitro and cell-based assays described above. The Muir laboratory is well equipped, both practically and intellectually, to conduct studies of this type (e.g. [95]).

We stress that the goal of this aim is to identify and then optimize tool compounds (global activators and inhibitors of *agr*) using various biochemical and cell-based assays. We are, however, cognizant that some of these modulators could have value as therapeutic leads. While beyond the scope of the current proposal, we do note that animal (murine) models of infection are available and have been used successfully to test our AIP inhibitors in the past [25, 47]. We envision this as being an exciting line of enquiry for the future.

SUMMARY: The accessory gene regulator (*agr*) locus found in all staphylococci encodes a quorum sensing (QS) circuit that controls the expression of virulence and other accessory genes. A research program will be undertaken to study *agr* signal transduction in the pathogen, *Staphylococcus aureus*. Building on recent breakthroughs that have allowed us to reconstitute much of the quorum sensing circuit using purified components, we will integrate chemical, biochemical, biophysical and genetic tools for the purpose of obtaining a deeper understanding into the molecular mechanisms underlying the production and sensing of the autoinducer peptide (AIP) pheromone that is central to *agr* regulation. The program will move forward in three directions, namely (i) identifying the key missing players in AIP biosynthesis, (ii) understanding how agonism and antagonism of the QS system relates to newly discovered conformational changes in the AIP receptor, AgrC, and (iii) identifying small molecule modulators of *agr* through sophisticated target-based screens. These studies will provide fundamental insights into how a QS circuit such as *agr* operates at the molecular level and will lay the foundation for the development of new strategies for treating *S. aureus* infections.

Vertebrate animals

1) Use of animals. The experiments to be conducted here are noted in the experimental approach section under Specific Aim 3.
Mice will be used as described below and tagged with luciferase as described in the proposal.
Animals to be used will be 8 or 12 week-old outbred Swiss-Webster mice of either sex.

General Methods

i) Mouse deep quadriceps (DQ) model. Three infecting doses, 10^7, 10^8, 10^9 bacteria + cytodex beads will be delivered in 25 µl PBS. Animals will be imaged daily to evaluate the persistence and localization of the infection. Twenty-seven mice will be used for this validation study.

ii) Mouse kidney abscess model. A reproducible kidney abscess-inducing dose will be determined by IV injection of graded doses of bacteria, 10^7, 10^8, 10^9 - 3 mice for each dose, evaluated by IVIS imaging daily for 5 days. Nine mice will be used for this validation study.

iii) Toxicity and pharmacokinetics. We propose to test the 4 most effective small molecules (SM1-4) for IV toxicity and to measure IV pharmacokinetics.

a) IV Toxicity: 500 µg/mouse (20 mg/kg). Mice will be monitored for signs of toxicity, including ataxic gate, decrease in activity, ruffled fur, weight loss, etc. 3 6-8 week old mice will be used per test, 12 total.

b) Pharmacokinetics: Three 12 week-old Swiss-Webster mice of either sex will be used for each experiment. Mice will be anesthetized and the femoral vein cannulated. The SM to be tested will be injected in 100 µl phosphate buffered normal saline (PBS) and 20 µl samples will be withdrawn for processing at 15 min intervals for 90 min.

iv) Testing for agr inhibition

a) Correlation between agr inhibition and attenuation of infection (DQ model). It is proposed to use 50 µg/mouse of the 4 most effective SMs in this test, 5 mice each for agr+ and agr-bacteria, and 5 untreated controls. If reproducible attenuation is not observed, we will use 4x this dosage (200 µg/mouse). Imaging will be performed at 24 h intervals for 5 days, after which mice will be euthanized and viable bacteria enumerated in tissue homogenates. 5 mice per group. Mice will be euthanized and viable counts compared with luciferase signals. Maximum number of mice - 90.

b) Efficacy of compound (kidney model). With the most effective SM and dose, we would verify efficacy in the kidney model, using USA300. This test would use 10 mice – 5 with the SM and 5 untreated.

c) Repeated dosage. With the most effective SM and dose, we will test for the effects of repeated dosage, administering the molecule at 0, 3 and 6 h after the bacteria. For this test, we will use USA300 in the DQ model and 10 mice.

d) Delayed administration. We will test for the effects of delayed administration of the best SM, using intervals of 0, 24 or 48 h between injection of bacteria and peptide. These tests will use the most effective SM with USA300 in the kidney model and will require 20 mice – 5 for each interval and 5 untreated.

v) Synergism with antibiotics. If the hypothesis that inhibiting agr attenuates an infection is not substantiated, we will test for SM synergy with vancomycin (vnc) in the DQ model. Each test would use 20 mice – 5 with SM, 2 mg/kg, 5 with vnc, 33 mg/kg, 5 with both, and 5 untreated, with treatment at 0, 24, or 48 h post infection. If the attenuation hypothesis is substantiated, the most effective agr inhibitor would again be tested for synergy with vnc. The SM and antibiotic will each be used alone at the same single doses as above, and in combination, using the same therapeutic regimen as above. The proposed experiments would utilize 40 mice. Testing of additional SMs would require 20 mice each. In the event that we are unable to identify a suitably effective small molecule by high-throughput screening, we will do essentially the same experiments as those proposed above with 4 different AIP derivatives: AIP-II, AIP-II N3A mutant, and the lactone derivatives of each. The maximum number of mice for both of the above sets of experiments would be 440.

2) Justification for the use of animals. The proposed studies are pre-clinical trials of therapeutic agents and

therefore can be done only with live animals. Although invertebrate models for studying staphylococcal infections are available, we do not believe that results obtained with such models could satisfactorily translate to the vertebrate organism. We generally use mice, since these are the “lowest” form of vertebrate suitable for such studies. It is noted, parenthetically, that our imaging system enables a 5-10-fold reduction in the numbers of animals required for our experiments, in comparison with methods that require point-by-point enumeration of bacteria.

3) Veterinary care. Animals are housed in micro isolator cages, 5 mice to a cage, in an animal room with proper ventilation, temperature control, and light-dark cycle, and are given a standard diet with water ad libitum.
Cages are changed weekly by the Skirball animal facility staff. Dead animals are autoclaved before discarding, or are dissected for the enumeration of viable bacteria in tissues and organs. In general, intravenous (IV) or intraperitoneal (IP) injections will be of 100 µl in PBS. Intramuscular (IM) injections will be of 25 µl of PBS. Moribund animals (ruffled fur, ataxic gait) will be euthanized by CO2 narcosis. For imaging, the animal will be anesthetized with isoflurane.

4) Minimization of pain and distress. Animals will be anesthetized with isoflurane for injections and for imaging. Infected animals will receive ibuprofen a dosage of 7.5 mg/kg, mixing the liquid form of Children's ibuprofen in the drinking water at that dosage. If an animal remains in discomfort despite the presence of ibuprofen in the drinking water, Buprenorphine at 0.05-0.1 mg/kg will be administered subcutaneously every 12 hours.

5) Euthanasia. Animals will be euthanized by CO2 narcosis, either at the end of an experiment or during an experiment if they show signs of severe morbidity. This is the standard method recommended by the AVMA.

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