PI: **STARNBACH, MICHAEL N**	Title: Alteration of host protein stability by Legionella	
Received: 10/18/2010	FOA: PA10-069	Council: 05/2011
Competition ID: ADOBE-FORMS-B	FOA Title: NIH EXPLORATORY DEVELOPMENTAL RESEARCH GRANT PROGRAM (PARENT R21)	
1 R21 AI096101-01	Dual:	Accession Number: 3338682
IPF: 3212902	Organization: HARVARD UNIVERSITY (MEDICAL SCHOOL)	
Former Number:	Department: Microbiology	
IRG/SRG: ZRG1 IDM-A (80)S	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 1: [redacted] Year 2: [redacted]	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:*
Michael Starnbach	President and Fellows of Harvard University	PD/PI

OMB Number: 4040-0001
Expiration Date: 06/30/2011

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

2. DATE SUBMITTED: 10/18/2010
Applicant Identifier: 5789461-01-5256928

4. a. Federal Identifier:
b. Agency Routing Identifier:

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

* Legal Name: President and Fellows of Harvard College
Department: Sponsored Programs Admin
Division: Harvard Medical School
* Street1: 25 Shattuck St
Street2:
* City: Boston
County / Parish:
* State: MA: Massachusetts
Province:
* Country: USA: UNITED STATES
* ZIP / Postal Code: 02115-6027

Person to be contacted on matters involving this application
Prefix: Ms.
* First Name: Deborah
Middle Name:
* Last Name: Good
Suffix:
* Phone Number: [redacted]
Fax Number: [redacted]
Email: [redacted]

6. * EMPLOYER IDENTIFICATION *(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: NIH

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER:
TITLE:

11. * DESCRIPTIVE TITLE OF APPLICANT'S PROJECT:
Alteration of host protein stability by Legionella

12. PROPOSED PROJECT:
* Start Date: 07/01/2011
* Ending Date: 06/30/2013

*** 13. CONGRESSIONAL DISTRICT OF APPLICANT**: MA-008

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION
Prefix: Dr.
* First Name: Michael
Middle Name: N.
* Last Name: Starnbach
Suffix:
Position/Title: Professor
* Organization Name: President and Fellows of Harvard University
Department: Microbiology
Division: Harvard Medical School
* Street1: Harvard Medical School
Street2: 200 Longwood Avenue
* City: Boston
County / Parish:
* State: MA: Massachusetts
Province:
* Country: USA: UNITED STATES
* ZIP / Postal Code: 02115-0000
* Phone Number: [redacted]
Fax Number: [redacted]
* Email: [redacted]

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested	[redacted]
b. Total Non-Federal Funds	0.00
c. Total Federal & Non-Federal Funds	[redacted]
d. Estimated Program Income	0.00

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 penalities. (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

Add Attachment | Delete Attachment | View Attachment

19. Authorized Representative

Prefix: * First Name: Kelly Middle Name: A.

* Last Name: Evans Suffix:

* Position/Title: Senior Sponsored Research Administrator/Train

* Organization: President and Fellows of Harvard College

Department: Sponsored Programs Admin Division: Harvard Medical School

* Street1: 25 Shattuck Street

Street2:

* City: Boston County / Parish:

* State: MA: Massachusetts Province:

* Country: USA: UNITED STATES * ZIP / Postal Code: 02115-6027

* Phone Number: [redacted] Fax Number: [redacted]

* Email: [redacted]

*** Signature of Authorized Representative**: Evans, Kelly A.

*** Date Signed**: 10/18/2010

20. Pre-application Add Attachment | Delete Attachment | View Attachment

424 R&R and PHS-398 Specific Table Of Contents

OMB Number: 4040-0010
Expiration Date: 08/31/2011

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: President and Fellow of Harvard College

DUNS Number: [redacted]

* Street1: 200 Longwood Avenue

Street2:

* City: Boston County: Suffolk

* State: MA: Massachusetts

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: 02115 * Project/ Performance Site Congressional District: MA-008

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:

DUNS Number:

* Street1:

Street2:

* City: County:

* State:

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: * Project/ Performance Site Congressional District:

Additional Location(s) Add Attachment Delete Attachment View Attachment

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

3. * Is proprietary/privileged information included in the application? ☐ Yes ☒ No

4.a. * Does this project have an actual or potential impact 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 of the United States or partnerships with international collaborators? ☐ Yes ☒ No

6.a. If yes, identify countries:

6.b. Optional Explanation:

7. * Project Summary/Abstract R21StarnbachProjectSummaryFinalOct10. Add Attachment | Delete Attachment | View Attachment

8. * Project Narrative R21StarnbachProjectNarrativeFinalOct1 Add Attachment | Delete Attachment | View Attachment

9. Bibliography & References Cited R21StarnbachLiteratureCitedFinalOct10 Add Attachment | Delete Attachment | View Attachment

10. Facilities & Other Resources R21StarnbachResourcesEnvironmentFinal Add Attachment | Delete Attachment | View Attachment

11. Equipment Add Attachment | Delete Attachment | View Attachment

12. Other Attachments Add Attachments | Delete Attachments | View Attachments ☐

Project Summary

Infection with the intracellular pathogen *Legionella pneumophila* can lead to a severe pneumonia known as Legionnaires' disease. *Legionella pneumophila* uses a specialized type IV secretion apparatus, also known as the Dot/Icm system, to secrete over 150 effector proteins directly into the host cell. The translocated bacterial effectors establish a vacuolar niche that supports replication of *L. pneumophila* in eukaryotic cells. While there is an extensive literature describing how several of these effectors alter host cell functions, the targets of most have remained elusive. A significant problem in linking a particular effector to a particular function is the redundant or overlapping activity of many effectors. This means that *L. pneumophila* mutant strains deficient in any one effector often have no appreciable phenotype, preventing the identification of their host targets. While it is well appreciated that many *L. pneumophila* effectors directly alter host proteins through functions such as E3 ubiquitin ligase activity, there have been few methods developed to monitor pathogen-induced changes in host protein stability on a large scale. Here we propose to apply a novel screening method called the "Global Protein Stability" (GPS) system to identify host cell proteins whose stability is altered by the secreted *L. pneumophila* effectors. Once we have identified host proteins that are stabilized or destabilized when a functional type IV secretion system is present, we will test whether reducing or increasing the prevalence of these proteins (attempting to reverse the effects of the *Legionella* effectors) impairs the capacity of *L. pneumophila* to replicate and survive within host cells. Once we identify which host proteins must be altered in order for *L. pneumophila* to replicate, we will take a targeted approach to identify which of the *L. pneumophila* effectors are causing these essential changes to host proteins. In addition, the GPS screen may also identify the targets of specific "families" of effectors that have remained elusive, such as the *L. pneumophila* E3 ubiquitin ligases. The directed approach we propose allows us to overcome the difficulties inherent in target identification, such as the redundancy of effectors, and identify the functions of effectors that have remained cryptic. Organism-induced alterations of the host are key to pathogenesis, yet it has previously not been possible to study alterations to individual host proteins at the scale the GPS system permits. The experiments described in this proposal allow, for the first time, dissection of how bacterial infection globally regulates host cell proteins and pathways beyond the transcriptional level.

Project Narrative

Legionella pneumophila, the causative agent of Legionnaires' disease replicates inside host cells. To manipulate the host cell and replicate intracellularly, the organism injects >150 of its proteins into host cells. The proposed research uses a large-scale approach to identify the targets of these injected bacterial proteins – identifying the host cell proteins that are destabilized or stabilized by the injected bacterial proteins. Once we identify which bacterial proteins are manipulating which host proteins, we can test methods to disrupt these interactions. This may lead to the development of new classes of antibiotics to treat bacterial infection.

RESOURCES

The PI's laboratory consists of 1700 square feet of space in the Warren Alpert Building. There is ample bench, desk space and a fume hood. In addition, we have exclusive access to a tissue culture room with four laminar flow hoods, a CO_2 incubator and microscopes. We have shared access to warm and cold rooms and several equipment rooms. A newly constructed SPF animal facility is located within the Warren Alpert Building. Space for infected mice is available in a newly constructed BL2/BL3 vivarium in an adjacent building. Apple Macintosh personal computers are located in both the office and laboratory space. All computers are attached by Ethernet to Harvard University computing facilities, the internet, software for analysis of sequence data, the Harvard Medical School library databases, and scientific literature databases.

The PI's office is 240 square feet and is attached to the laboratory space.

Administrative support, computer support, statistical consulting, bioinformatics support and secretarial support for this project is available in the Department of Microbiology and Molecular Genetics.

The equipment necessary to perform the research in this proposal is located either within our laboratory, within the Department of Microbiology & Molecular Genetics, or within the adjacent Immune Diseases Institute. These include centrifuges, FACScan and FACSaria flow cytometers/sorters, oligonucleotide synthesizers, thermal cyclers, HPLC, refrigerators, freezers, -80^0 C freezer, liquid N_2 storage, and electrophoresis equipment.

The Department of Microbiology and Molecular Genetics has just purchased an Agilent array reader that is available for analyzing the arrays in this project.

SCIENTIFIC AND INTELLECTUAL ENVIRONMENT

To succeed in the proposed research, we will need to combine classical and molecular genetics, biochemistry, immunology, cell biology, and conventional and high-resolution microscopy. The location of our laboratory within the Department of Microbiology and Molecular Genetics and within the wider community of Harvard Medical School (HMS) is ideally suited for maximizing our chances of success. The high density of scientific leaders and innovators with well-equipped facilities at HMS has made it possible to assemble a group of outstanding colleagues to provide critical support and advice for all aspects of the proposed project. The most important of these interactions will be with Dr. Stephen Elledge in our Department of Genetics. We also will formally consult on a quarterly basis with a number of Harvard Medical School colleagues including Drs. Dan Finley and Randy King (experts on protein turnover and the ubiquitin-proteasome pathway), Drs. Eric Rubin and Stephen Lory (who have expertise on genomic approaches to the study of bacterial pathogenesis), and Dr. Deb Hung (a chemical biologist interested in new approaches to develop antimicrobial compounds).

In addition to these outstanding colleagues, the Department of Microbiology and Molecular Genetics has a long tradition of collegiality, active scientific exchange, and collaboration. The week is bracketed by scientific and social events that bring the entire community of faculty and trainees together. Every Monday we have student-postdoc research presentations (two talks, 25 minutes each) and every Friday we have beer hour. Both events are equally well-attended and promote scientific exchange and seed new collaborations. In addition, the department hosts a weekly seminar series where leading researchers in the fields of microbiology, cell biology, and immunology are invited to present current research in these areas. Our research group also attends the weekly Immunology program seminar and social hour.

OMB Number: 4040-0001
Expiration Date: 06/30/2011

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: Dr. * First Name: Michael Middle Name: N.
* Last Name: Starnbach Suffix:
Position/Title: Professor Department: Microbiology
Organization Name: President and Fellows of Harvard University Division: Harvard Medical School
* Street1: Harvard Medical School
Street2: 200 Longwood Avenue
* City: Boston County/ Parish:
* State: MA: Massachusetts Province:
* Country: USA: UNITED STATES * Zip / Postal Code: 02115-0000
* 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: Ph.D.
Degree Year: 1992

***Attach Biographical Sketch** R21StarnbachBioOCT2010ABSFINA [Add Attachment] [Delete Attachment] [View Attachment]
Attach Current & Pending Support [Add Attachment] [Delete Attachment] [View Attachment]

PROFILE - Senior/Key Person 1

Prefix: * First Name: Middle Name:
* Last Name: Suffix:
Position/Title: Department:
Organization Name: Division:
* Street1:
Street2:
* City: County/ Parish:
* State: Province:
* Country: USA: UNITED STATES * Zip / Postal Code:
* Phone Number: Fax Number:
* E-Mail:

Credential, e.g., agency login:

*** Project Role:** **Other Project Role Category:**

Degree Type:
Degree Year:

***Attach Biographical Sketch** [Add Attachment] [Delete Attachment] [View Attachment]
Attach Current & Pending Support [Add Attachment] [Delete Attachment] [View Attachment]

BIOGRAPHICAL SKETCH

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

NAME	POSITION TITLE
Michael Starnbach	Professor
eRA COMMONS USER NAME [redacted]	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Vassar College, Poughkeepsie, NY	A.B	1987	Biology
Stanford University, Stanford, CA	Ph.D	1992	Micro. & Immuno.
University of Washington, Seattle, WA	None		Postdoctoral Research

A. Personal Statement

Research in my lab uses a combination of cellular and molecular approaches in the analysis of host responses to bacterial pathogens. Many virulence factors have been identified that allow bacteria to survive and replicate within the mammalian host and often within host cells. I have focused on the consequences of these survival strategies, specifically asking how bacterial virulence factors subvert or inhibit host responses allowing for chronic infection. My lab has led the efforts to dissect antigen specific T cell responses to chronic bacterial infection, including *C. trachomatis*. Approximately half the members of my research team are cellular immunologists focused on the T cell response to bacterial pathogens. The other half of my research team focuses on bacterial pathogenesis, with a particular interest in how bacterial virulence determinants modify host cells and how host cell innate immune pathways allow for resistance to intracellular growth. Recent work on cellular factors needed to constrain bacterial growth has used *Legionella pneumophila* as the model pathogen, so my lab is already prepared to conduct these studies. Working at the interface of bacterial pathogenesis and cellular immunology reflects my prior training, with graduate work focused on bacterial virulence determinates and postdoctoral work in T cell immunobiology.

B. Positions and Honors.

1985-1987 Research Assistant; Vassar College with E. Pinina Norrod, Department of Biology
1987-1992 Graduate student and research assistant, Stanford University School of Medicine with Stanley Falkow, Department of Microbiology and Immunology
1990-1992 Research *in absentia*. Laboratory of Stephen Lory, University of Washington School of Medicine, Department of Microbiology
1992-1995 Associate, Howard Hughes Medical Institute, and Senior Fellow, Department of Immunology; University of Washington School of Medicine, with Michael Bevan.
1995-2001 Assistant Professor, Dept. of Microbiology and Molecular Genetics, Harvard Medical School.
1997-date Tutor in Biochemical Sciences, Harvard University.
2001-2008 Associate Professor, Dept. of Microbiology and Molecular Genetics, Harvard Medical School
2008-date Professor, Dept. of Microbiology and Molecular Genetics, Harvard Medical School

Academic Honors

1987 Departmental Honors in Biology and General Honors, Vassar College
1987-1989 Departmental USPHS Predoctoral Traineeship, Stanford University
1989-1990 Cellular and Molecular Biology USPHS Predoctoral Traineeship, Stanford University
1996-1999 Junior Faculty Research Award, American Cancer Society

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

1. Starnbach, M. N, and M. J. Bevan. 1994. Cells Infected with *Yersinia* Present an Epitope to Class I MHC Restricted Cytotoxic T-Lymphocytes. *Journal of Immunology* 153:1603-1612.

2. Starnbach, M. N, M. J. Bevan, and M. F. Lampe. 1994. Protective Cytotoxic T-Lymphocytes are induced during Murine Infection with *Chlamydia trachomatis*. *Journal of Immunology*, 153:5183-5189.

3. Ballard, J. D., R. J. Collier, and M. N. Starnbach. 1996. Anthrax toxin mediated delivery of a cytotoxic T-cell peptide epitope *in vivo. Proceedings of the National Academy of Sciences, USA*, 93:12531.

4. Lampe, M. F., M. J. Bevan, C. B. Wilson, and M. N. Starnbach. 1998. Gamma interferon production by cytotoxic T-lymphocytes is required for resolution of *Chlamydia trachomatis* infection. *Infection and Immunity*, 66:5457-5461.

5. Fling, S., A. Sutherland, L. Steele, B. Hess, S. D'Orazio, J-F. Maisonneuve, M. Lampe, P. Probst, and M.Starnbach. 2001. CD8+ T-cells Recognize a Novel Inclusion Membrane Associated Protein from the Vacuolar Pathogen *Chlamydia trachomatis*. *Proceedings of the National Academy of Sciences, USA*, 98:1160-1165.

6. Boyartchuk V.L., K.W. Broman, R.E. Mosher, S.E. D'Orazio, M.N. Starnbach, and W.F. Dietrich. 2001. Multigenic control of *Listeria monocytogenes* susceptibility in mice. *Nature Genetics*, 27:259-60.

7. Starnbach, MN, Loomis WP, Ovendale P, Regan D, Hess B, Alderson MR, Fling SP. An inclusion membrane protein from *Chlamydia trachomatis* enters the MHC class I pathway and stimulates a $CD8^+$ T cell response. Journal of Immunology, 2003;171:4742-4749.

8. Ho, TD, Starnbach, MN. The *Salmonella*-encoded type III secretion system can translocate *Chlamydia* proteins. Infection and Immunity, 2005;73:905-911.

9. Bernstein-Hanley, I, Balsara, ZR, Ulmer, W, Coers, J, Starnbach, MN, Dietrich, WF. Genetic analysis of susceptibility to Chlamydia trachomatis in mouse, Genes and Immunity, 2006, 7:122-129.

10. Misaghi, S, Balsara, ZR, Catic, A, Spooner, E, Ploegh, HL, Starnbach, MN. Chlamydia trachomatis-derived deubiqitinating enzymes in mammalian cells during infection, Molecular Microbiology, 2006, 61:42-50.

11. Bernstein-Hanley, I, Coers, J, Balsara, ZR, Taylor, GA, Starnbach, MN, Dietrich, WF. The p47 GTPases IGTP and Irgb10 map to the Chlamydia trachomatis susceptibility locus Ctrq-3 and mediate cellular resistance in mouse. Proceedings of the National Academy of Sciences, USA, 2006, September 7.

12. Balsara, ZR, Misaghi, S, Lafave, JN, Starnbach, MN. Chlamydia trachomatis infection induces cleavage of the mitotic cyclin B1. Infection and Immunity, 2006, 74:5602-5608.

13. Coers, J, Bernstein-Hanley, I, Grotsky, D, Parvanova, I, Howard, JC, Taylor, GA, Dietrich, WF, Starnbach, MN. *Chlamydia muridarum* evades growth restriction by the IFN□ inducible host resistance factor Irgb10. Journal of Immunology, 2008, 180:6237-6245.

14. Henry, SC, Daniell, XG, Burroughs, AR, Indaram, M, Howell, DN, Coers, J, Starnbach, MN, Hunn, JP, Howard, JC, Feng, CG, Sher, A, Taylor, GA. Balance of Irgm protein activities determines IFNg-induced host defence. J Leukoc Biol. 2009 Jan 27. PMC ID: PMC2669409.

15. Coers, J, Starnbach, MN, Howard, JC. Modeling infectious disease in mice: co-adaptation and the role of host-specific IFNγ responses, PLOS Pathogens, May 29, 2009. PMC ID: PMC2682201.

D. Research Support.

Ongoing Research Support

R01 AI039558 Starnbach (PI) 6/1/06 - 5/31/11
NIH/NIAID
Cytotoxic T cell Mediated Immunity to *Chlamydia*
The major goals of this project are to identify C. *trachomatis* genes encoding CD8+ T cell epitopes, test their efficacy in a model vaccine, and define the mechanism of CD8+ T cell protection.
Role: PI

R01 AI062827 Starnbach (PI) 2/01/06 – 1/31/11
NIH/NIAID
Genetics of Innate Immunity to *Chlamydia trachomatis*
The goal of this project is to identify mouse loci associated with susceptibility and/or resistance to *C. trachomatis* infection.
Role: PI

R01 AI055962 Starnbach (PI) 6/1/09 – 5/31/11
NIH/NIAID
Inhibition of T cell Responses by Bacteria
The major goal of this project is to identify and characterize the defect in MHC-I processing and/or presentation that occurs during S. *flexneri* infection.
Role: PI

Completed Research Support

None.

PHS 398 Cover Page Supplement

OMB Number: 0925-0001

1. Project Director / Principal Investigator (PD/PI)

Prefix: Dr.
* First Name: Michael
Middle Name: N.
* Last Name: Starnbach
Suffix:

2. Human Subjects

Clinical Trial? ☐ No ☐ Yes

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

3. Applicant Organization Contact

Person to be contacted on matters involving this application

Prefix: Ms.
* First Name: Deborah
Middle Name:
* Last Name: Good
Suffix:

* Phone Number: [redacted]
Fax Number: [redacted]
Email: [redacted]

* Title: Associate Director

* Street1: 25 Shattuck Street
Street2:
* City: Boston
County/Parish:
* State: MA: Massachusetts
Province:
* Country: USA: UNITED STATES
* Zip / Postal Code: 02115-6027

PHS 398 Cover Page Supplement

4. Human Embryonic Stem Cells

* Does the proposed project involve human embryonic stem cells? ☒ No ☐ Yes

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://stemcells.nih.gov/research/registry/. 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:

Cell Line(s): ☐ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

Tracking Number:GRANT10719593 Funding Opportunity Number:PA-10-069 Received Date:2010-10-18T11:18:16-04:00

PHS 398 Modular Budget, Periods 1 and 2

OMB Number: 0925-0001

Budget Period: 1

Start Date: 07/01/2011 End Date: 06/30/2012

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	[redacted]
Consortium F&A	0.00
* Total Direct Costs	[redacted]

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	MTDC Organized Research (A02)	[redacted]	[redacted]	[redacted]
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS , Robert Aaronson , 212.264.2069

Indirect Cost Rate Agreement Date: 04/28/2010

Total Indirect Costs: [redacted]

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($): [redacted]

Budget Period: 2

Start Date: 07/01/2012 End Date: 06/30/2013

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	[redacted]
Consortium F&A	0.00
* Total Direct Costs	[redacted]

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	MTDC Organized Research (A02)	[redacted]	[redacted]	[redacted]
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS , Robert Aaronson , 212.264.2069

Indirect Cost Rate Agreement Date: 04/28/2010

Total Indirect Costs: [redacted]

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($): [redacted]

PHS 398 Modular Budget, Periods 3 and 4

Budget Period: 3

Start Date: End Date:

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	
Consortium F&A	
* Total Direct Costs	

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.				
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number)

Indirect Cost Rate Agreement Date

Total Indirect Costs

C. Total Direct and Indirect Costs (A + B) Funds Requested ($)

Budget Period: 4

Start Date: End Date:

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	
Consortium F&A	
* Total Direct Costs	

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.				
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number)

Indirect Cost Rate Agreement Date

Total Indirect Costs

C. Total Direct and Indirect Costs (A + B) Funds Requested ($)

PHS 398 Modular Budget, Periods 5 and Cumulative

Budget Period: 5

Start Date: End Date:

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	
Consortium F&A	
* Total Direct Costs	

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.				
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number)

Indirect Cost Rate Agreement Date

Total Indirect Costs

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($)

Cumulative Budget Information

1. Total Costs, Entire Project Period

*Section A, Total Direct Cost less Consortium F&A for Entire Project Period	$ [redacted]
Section A, Total Consortium F&A for Entire Project Period	$ 0.00
*Section A, Total Direct Costs for Entire Project Period	$ [redacted]
*Section B, Total Indirect Costs for Entire Project Period	$ [redacted]
*Section C, Total Direct and Indirect Costs (A+B) for Entire Project Period	$ [redacted]

2. Budget Justifications

Personnel Justification	R21StarnbachBudgetJustificatio	Add Attachment	Delete Attachment	View Attachment
Consortium Justification		Add Attachment	Delete Attachment	View Attachment
Additional Narrative Justification		Add Attachment	Delete Attachment	View Attachment

BUDGET JUSTIFICATION

PERSONNEL

Michael Starnbach (Principal Investigator, 1.2 Calendar months). Michael Starnbach will be responsible for the overall intellectual direction of this project. He will supervise and be responsible for the conduct of all the studies in this proposal, review all protocols and data, and supervise and train all other personnel.

Catarina Nogueira (Post-Doc, 12 Calendar months). Catarina Nogueira will take primary responsibility for the GPS screens (Aim 1) and the identification of the bacterial effectors responsible (Aim 2). Dr. Nogueira is an expert in how bacteria modify host proteins, having conducted doctoral work in Craig Roy's lab at Yale where she studied *L. pneumophila*'s capacity to manipulate host regulatory pathways to induce apoptosis.

Hannah Ratcliffe (Research Assistant, 3 Calendar months). Hannah Ratcliffe will assist Dr. Nogueira with the extensive cell culture and molecular biology required for this project. She will also maintain tissue culture supplies and reagents.

OMB Number: 0925-0001

PHS 398 Research Plan

1. Application Type:

From SF 424 (R&R) Cover Page. The response provided on that page, regarding the type of application being submitted, is repeated for your reference, as you attach the appropriate sections of the Research Plan.

*Type of Application:

☒ New ☐ Resubmission ☐ Renewal ☐ Continuation ☐ Revision

2. Research Plan Attachments:

Please attach applicable sections of the research plan, below.

1. Introduction to Application (for RESUBMISSION or REVISION only)		Add Attachment	Delete Attachment	View Attachment
2. Specific Aims	R21Starnbach2SpecificAimsFin	Add Attachment	Delete Attachment	View Attachment
3. *Research Strategy	R21Starnbach3ResearchStrate	Add Attachment	Delete Attachment	View Attachment
4. Inclusion Enrollment Report		Add Attachment	Delete Attachment	View Attachment
5. Progress Report Publication List		Add Attachment	Delete Attachment	View Attachment

Human Subjects Sections

6. Protection of Human Subjects		Add Attachment	Delete Attachment	View Attachment
7. Inclusion of Women and Minorities		Add Attachment	Delete Attachment	View Attachment
8. Targeted/Planned Enrollment Table		Add Attachment	Delete Attachment	View Attachment
9. Inclusion of Children		Add Attachment	Delete Attachment	View Attachment

Other Research Plan Sections

10. Vertebrate Animals		Add Attachment	Delete Attachment	View Attachment
11. Select Agent Research	R21StarnbachSelectagentsOct	Add Attachment	Delete Attachment	View Attachment
12. Multiple PD/PI Leadership Plan		Add Attachment	Delete Attachment	View Attachment
13. Consortium/Contractual Arrangements		Add Attachment	Delete Attachment	View Attachment
14. Letters of Support	R21StarnbachElledgeSupportLe	Add Attachment	Delete Attachment	View Attachment
15. Resource Sharing Plan(s)	R21Starnbachresourcesharing0	Add Attachment	Delete Attachment	View Attachment

16. Appendix Add Attachments | Remove Attachments | View Attachments

List of Research Plan Attachments

2. Specific Aims

L. pneumophila is a powerful model organism that can be used to understand the interactions between intracellular pathogens and the host cells they infect. Our lab has been interested in understanding, at a global scale, how effector proteins that are translocated into host cells during bacterial infection post-translationally manipulate host cell proteins and pathways. Although some host cell proteins targeted by specific *L. pneumophila* type IV secreted effectors have been identified, most have remained elusive. We have been working with Stephen Elledge's lab in the Department of Genetics to adapt their recently-developed "Global Protein Stability" (GPS) system to analyze how translocated effector proteins impact the stability of individual host cell proteins. Our goal in this proposal is to globally characterize changes in host protein stability caused by translocated proteins and to begin identifying specific *L. pneumophila* effectors responsible for these alterations. Understanding on a large scale what host proteins are altered by specific bacterial effectors will allow us to uncover host pathways that are critical for *L. pneumophila* replication and survival inside a cell. Specifically, we will:

Specific Aim 1. Identify and characterize host cell proteins that are stabilized or destabilized by *L. pneumophila* translocated effectors. To better understand how *L. pneumophila* manipulates host cells, we will conduct a "Global Protein Stability" (GPS) screen to identify host proteins that are altered in stability when the *Legionella* type IV secretion system is present. We will determine the stability of >12,000 individual host proteins during infection with wild-type *L. pneumophila* and then identify which of these host proteins are more or less stable when the cell is infected with *L. pneumophila* (*ΔdotA*) that lacks a functional type IV secretion system (and is unable to secrete effectors into cells). Once we identify proteins whose stability is altered by the *L. pneumophila* effectors, we will determine whether reversing these changes, by either reducing or increasing the prevalence of these host proteins, impairs the intracellular growth of *L. pneumophila*. This will identify host proteins whose stability must be manipulated by *L. pneumophila* in order for the organism to grow.

Specific Aim 2. Determine the *L. pneumophila* Dot/Icm-translocated substrates that are responsible for alterations in host protein stability. Previous loss-of-function studies using *L. pneumophila* mutants have been unable to identify the targets of many effectors due to significant redundancy in the function of the effectors. Once we identify changes in host protein stability mediated by the translocated effectors (in Specific Aim 1), we can then test which *L. pneumophila* effector(s) is responsible for that alteration. This allows a more direct approach to identify bacterial effector/host target pairs important in pathogenesis. Together these approaches will help us understand both the host proteins that are altered in stability and the bacterial effectors responsible for these changes. If these effector-mediated manipulations of host cell protein stability are required for growth, it will allow us to develop new classes of antibiotics that prevent these manipulations and prevent bacterial growth.

Through these two fundamentally linked aims we will use GPS to identify host proteins, pathways or networks altered by *L. pneumophila* translocated effectors. We then can begin to identify the specific bacterial virulence factors responsible for those alterations. The use of GPS is an innovative method that can be adapted widely to understand host-pathogen interactions.

3. Research Strategy

3(a). Significance. Intracellular bacterial pathogens have evolved to exploit host cells in order to survive within and spread from mammalian hosts. Many of these bacterial pathogens reside in vacuoles and translocate virulence factors into the host cell cytosol through specialized secretion systems in order to avoid destruction by the endocytic pathway and allow replication within the cell (12). The activities of secreted effectors and their impact on host cell functions is an area of intense investigation. To completely understand how bacteria impact, exploit, or affect host cell functions during infection, one must study multiple aspects of host cell protein regulation during infection, including transcriptional regulation, regulation of translation, as well as protein modification, localization and turnover. Much research activity over the past decade has focused on the opportunities available to study transcriptional regulation of host cell functions during infection. The ability to screen arrays of host cell genes during bacterial infection has led to an explosion in the available data showing which mammalian genes are induced and repressed as a result of infection. However, bacterial manipulations of host cell proteins (for example the activity of bacterial proteases) (18) cannot be discovered by monitoring transcription. It simply has not been previously possible to examine how bacterial infection directly affects the stability of individual host proteins at the scale afforded by transcriptional arrays. *L. pneumophila* has served as an exceptional model pathogen for dissecting complex changes in host cell biology that occur during infection. Especially well-characterized are the protein effectors secreted by the *L. pneumophila* type IV secretion system. Here we propose to conduct a "Global Protein Stability" (GPS) screen to determine the impact of *Legionella* type IV secretion on the stability of >12,000 individual host cell proteins. To accomplish this, we will identify proteins whose stability differs when cells are infected with wild-type *L. pneumophila* vs. a mutant strain (*ΔdotA*) that lacks a functional type IV secretion system. This approach will allow us to identify a significant subset of host cell proteins whose stability is altered by the *L. pneumophila* type IV effectors. Once we have identified proteins whose stability is altered during infection, we will conduct experiments to determine whether some of these host protein alterations are necessary for intracellular growth of *L. pneumophila*. At the same time, we will also work to identify which *L. pneumophila* effector proteins are responsible for individual protein changes in the host. As a longer-term goal, we would like to target with experimental therapeutics the host proteins that are required for successful bacterial replication – developing novel classes of "host-based" antibiotics.

The ability to identify, study and target host proteins altered by microbial infection will be compelling to most cellular microbiologists regardless of the microbe they study. Therefore we are confident that GPS will be widely adopted to study the pathogenesis of infectious disease caused by bacteria, viruses and parasites.

3(b). Innovation. Although most investigators studying the effects of bacteria on host cells appreciate that gene regulation and protein turnover are both key to understanding how bacteria alter host cell functions, there has been a bias towards the study of gene regulation rather than protein stability. This bias is a result of the availability of arrays and other tools that allow the simultaneous measurement of thousands of individual host cell gene transcripts during a particular stage of cell infection. Although proteomics is an active area of investigation, the ability to monitor changes in protein abundance globally is limited. Approaches to study protein levels usually involve pulse-chase experiments, the administration of protease inhibitors followed by biochemical analysis, or biochemically trapping individual protein-protein interactions. These approaches are inherently limited in the number of proteins that can be monitored at one time, and are not scalable to make them more "global". We are interested in understanding how *L. pneumophila* type IV secreted effectors manipulate host cell protein function and turnover at a global level, and to determine whether some of these manipulations are required for intracellular replication. It is known that the type IV (Dot/Icm) secretion apparatus is crucial for *L. pneumophila* survival and replication inside eukaryotic cells. Over 150 Dot/Icm effector proteins have been identified through a variety of genetic, biochemical and cell biological approaches, yet for most of these proteins the biochemical functions and specific host targets remain unknown (4, 10, 16). We believe that many of the *L. pneumophila* effector proteins secreted during bacterial infection alter host cell proteins and pathways and allow the bacteria to establish a replicative niche. However, we have been frustrated by the inability to study changes in host cell protein stability at a scale provided by transcriptional arrays. The GPS screens being developed by our colleagues in the Genetics department are a novel, highly innovative method to globally monitor protein stability in mammalian cells, and we felt it could be adapted as a completely new large-scale approach to identify host proteins altered during bacterial infection. As proof-of-principle, we have begun to use GPS to globally monitor the stability of host proteins altered by *L. pneumophila* effectors during infection. GPS has the potential to profoundly increase our knowledge of the basic science of protein regulation during *L. pneumophila* infection and significantly increase our potential to target and cure this disease. Importantly, this method is applicable to most other classes of microbial pathogens and it is likely to identify significant virulence mechanisms not previously detectable using previously available methods. It isn't

hard to imagine that GPS may revolutionize the study of how bacterial infection globally regulates host cell proteins and pathways beyond the transcriptional level.

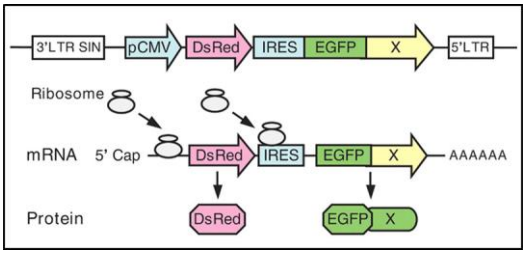
3(c). Approach (Specific Aim 1). Identify and characterize host cell proteins that are stabilized or destabilized by *L. pneumophila* translocated effectors.

3(c).i. Modification of host cell proteins by Dot/Icm-dependent secreted effectors is required for *L. pneumophila* intracellular replication. *L. pneumophila* is a facultative intracellular pathogen that is present in fresh-water reservoirs (5). Inhalation of aerosolized water droplets contaminated with *L. pneumophila* results in bacterial uptake by alveolar macrophages that support bacterial replication (9). Without antibiotic therapy, bacterial replication can lead to the development of a severe pneumonia known as Legionnaires' disease (6). As the use of artificial water reservoirs, such as air conditioning units and other man-made devices capable of aerosolizing contaminated water have become more common, epidemics and pneumonia caused by *L. pneumophila* have also become more prevalent (23).

To establish replication in its vacuolar niche, *L. pneumophila* inhibits lysosomal fusion and remodels its membrane-bound compartment into an organelle that resembles the endoplasmic reticulum (ER) (8). The ability of *L. pneumophila* to replicate in a ER-like compartment is dependent on a type IV secretion system, also known as the Dot/Icm secretion apparatus (19, 22). Over 150 Dot/Icm substrates have been identified, but for most of these proteins, the specific effector activities remain unknown (4, 10, 16). Using GPS, we can determine the stability of >12,000 host proteins during infection with wild-type *L. pneumophila*, and then identify which of these host proteins are more or less stable during infection with a *dotA L. pneumophila* mutant strain that lacks a functional Dot/Icm secretion system (and therefore translocates no effectors). This comparison of wild-type and mutant *L. pneumophila* strains will provide a vastly enhanced perspective on the targeting of host cell proteins by *L. pneumophila*-translocated substrates and the extent to which this targeting is required for infection, replication, and dissemination.

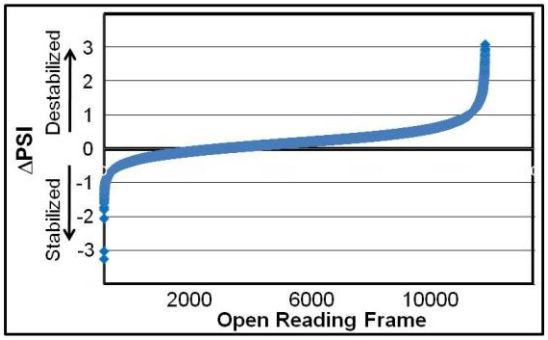
The "Global Protein Stability" (GPS) system is a highly innovative method developed by the Elledge lab at Harvard Medical School to globally monitor changes in protein stability mediated by host cell ubiquitin ligases (24, 25). In our lab we are adapting this method as a novel approach to understand host-pathogen interactions. Here we propose to apply the GPS system to understand the extent to which *L. pneumophila* Dot/Icm-dependent virulence factors modulate host cellular functions by stabilizing and destabilizing host proteins during infection.

3(c).ii. Global Protein Stability (GPS) profiling of *L. pneumophila*-infected mammalian cells. The Elledge lab recently published a description of the GPS platform as a method to identify the substrates of a human E3 ubiquitin ligase (24, 25), and we have now applied that system to identify proteins whose stability is altered by *L. pneumophila* secreted effectors. The Elledge lab provided to us a library of HEK 293T cells each containing a single copy of an expression cassette fused to a different human ORF.



The pooled library of HEK 293T cells we obtained was assembled from an arrayed set (ORFeome v3.1) of 12,000 human ORFs, each engineered into a unique retroviral reporter construct whose general structure is shown in the figure above. The reporter expression cassette contains a single pCMV promoter that with an internal ribosome entry site (IRES) permits the translation of two fluorescent proteins from one mRNA transcript. The first fluorescent protein is DsRed and the second is an enhanced GFP (EGFP) expressed as a fusion to one of 12,000 host cell proteins (EGFP-X). When introduced into the genome of cells, DsRed and EGFP-X should be produced at a constant ratio (independent of normal transcriptional regulation) because they are translated from the same mRNA (and regardless of where the construct integrates). However, while the amount of DsRed remains constant, the level EGFP becomes subject to the stability of the protein fused to its C-terminus. Therefore for each fusion construct in the library a baseline EGFP:DsRed ratio (reflecting relative stability) can be measured using fluorescent activated cell sorting (FACS). In our approach, we infected one copy of the library with wild-type *L. pneumophila*, and infected another identical copy with *L. pneumophila* Δ*dotA*. For any host protein whose stability is altered by a Dot/Icm-dependent secreted effector, there will be a dramatic difference in the EGFP:DsRed ratio in cells expressing that ORF between the two libraries. A differential change in EGFP:DsRed ratio will occur between corresponding ORFs in the two libraries anytime one or more Dot/Icm-dependent effector changes the stability of the EGFP-X fused to that ORF. For example, the effector may induce or deplete proteins that regulate the stability of X, or an effector may directly stabilize X, destabilize X, or cleave X. Controls conducted by the Elledge lab (25) have shown that the N-terminal EGFP fusion almost never alters the stability of the protein to which it is fused. Controls in our lab have shown that *L. pneumophila* replicates robustly in HEK 293T cells and that infection of these cells with *L. pneumophila* does not alter the inherent stability of DsRed or EGFP when expressed alone.

In our experiments, based on the published methods of the Elledge lab and incorporating all of the controls described (24, 25)), HEK 293T cells were infected for 24 h with wild-type *L. pneumophila* and sorted by high-throughput FACS into 7 bins (sorting 1 million cells into each bin). Cells were sorted into the bins based on their individual EGFP:DsRed ratio, with increasing EGFP:DsRed ratios corresponding to bins 1-7 respectively. Because sorting of cells into the distinct bins was dependent on the stability of the individual EGFP-X they express, the stability of an individual EGFP-X can be inferred from the distribution of cells expressing that particular EGFP-X within the 7 bins (abundance in bin 7 indicating stability of X, abundance in bin 1 indicating instability of X). The ORFs (Xs) in each bin were then amplified in bulk using PCR and labeled with Cy3 using an *in vitro* transcription kit. The ORFs (Xs) in the pre-sorted library were also amplified in bulk using PCR and labeled with Cy5 using an *in vitro* transcription kit. Because the labeled material is derived from genomic DNA, the labels represent the presence of an ORF within the bin or overall library, and are completely independent of transcription. The Cy3 labeled amplified ORFs from bin 1 were then mixed 1:1 with the Cy5 labeled amplified ORFs from the pre-sort library and applied to a custom microarray containing probes for the coding region of the 12,000 host proteins comprising the library. For each of the host protein ORF probes represented on the microarray, the ratio of Cy3:Cy5 indicates the prevalence of cells bearing that ORF in that bin. The amplified ORFs from bins 2-7 were similarly prepared and applied to a total of 7 microarrays. By compiling Cy3:Cy5 ratio data from all 7 arrays, we were able to characterize the prevalence of cells bearing a particular ORF in each of the seven bins. The presence of a cell bearing a particular ORF in higher number bins indicated that that protein is stable in the cells; the presence of a cell bearing a particular ORF in lower number bins indicated that that protein is unstable in the cells. To represent the protein stability information from the arrays quantitatively we calculated a protein stability index (PSI) for each of the host proteins in the library. PSI values for each host protein range from 1 to 7 with a higher PSI indicating relative protein stability. The calculation of PSI is described in the published report (25), but essentially identifies in which bin the population of cells bearing a particular ORF "peaks".

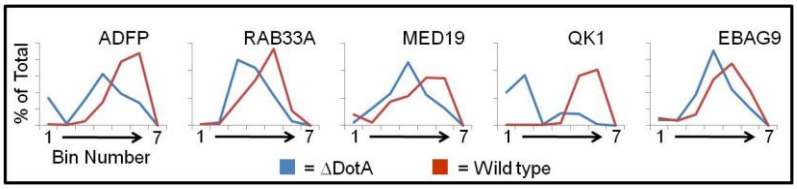


In parallel to the experiment described above, we infected the library with the *L. pneumophila dotA* mutant strain. We then sorted this library into 7 bins as above, amplified the ORFs from each bin, and hybridized them to 7 microarrays. We calculated a PSI for each ORF for the *ΔdotA* infection. We then compared the PSI value we obtained for each host protein in the library during infection with wild-type with the PSI value we obtained during infection with the *dotA* mutant. The subtraction of the "wild-type" PSI value from the "*ΔdotA*" PSI value gives us a ΔPSI. ΔPSI expresses the impact of Dot/Icm-secreted effector/s on any particular ORF. The theoretical range of ΔPSI values varies from -6 (the theoretical limit of proteins most "stabilized" as a result of the *dotA* mutation) to +6 (the theoretical limit of proteins most "destabilized" as a result of the *dotA* mutation). The figure above is a graph representing the ΔPSI values from our preliminary run.

3(c).iii. Notes on the arrays and hybridization controls used in the Global Protein Stability (GPS) profiling of *L. pneumophila*-infected mammalian cells. The custom microarrays used in the GPS system were designed by the Elledge lab and are manufactured by Agilent. The arrays contain over 44,000 different oligonucleotide probes of uniform length. The probes correspond to the coding region of the 12,000 host cell ORFs in the ORFeome v3.1 library. The number of probes per ORF ranges from 1 to 5 and the number of probes was designed to reflect the length of the ORF. Because most ORFs will hybridize to more than one probe, we can determine the consistency of the arrays and our hybridization procedures within any single array. These internal hybridization controls have proven remarkably consistent. Additionally, we can control across arrays by comparing the hybridization of the Cy5 labeled pre-sort ORFs between arrays. This has also been remarkably consistent. As an additional control across arrays we always spike the ORF mix with control sequences from Agilent labeled with Cy3 and Cy5 at predetermined ratios. The spike-in control sequences correspond to control probes incorporated into all Agilent microarrays.

Even though the GPS system in its current form has yielded very exciting results, the dependence of the system on multiple microarrays may produce some noise and limit resolution. We are now working in collaboration with scientists at the Harvard/MIT Broad Institute to develop Illumina based sequencing methods. Once developed, this will provide us with deep sequence-based quantitation for every bin in our GPS runs, enhancing the resolution of each screen greatly. Although a similar method of deep sequencing has been used to identify essential *Haemophilus* genes *in vivo* using "transposon capture and sequencing" (TraCS, which inspired us to explore the use of deep sequencing for our work) (7), this will be the first example of deep sequencing being used to identify the impact of bacterial virulence factors on host proteins.

3(c).iv. Small-scale validation of host proteins that are predicted by GPS to be stabilized by *L. pneumophila* Dot/Icm secreted effectors. As a first assessment of our initial GPS screen, we focused on host proteins that were stabilized in the presence of a functional Dot/Icm system. To optimize our initial selection, we generated graphs for several hundred of the proteins predicted to be most stabilized (most negative ΔPSI) from our GPS runs. The graphs represent the distribution of cells bearing that ORF over bins 1-7 as determined by the microarray hybridization - showing the results when cells bearing that ORF were either infected with wild-type *L. pneumophila* (blue) or the *ΔdotA* strain (red). Several examples are shown in the figure. For each of these graphs, a clear shift is observed in the "peak" bin when a functional Dot/Icm system is present. Again, the examples in the figure reflect preliminary GPS screen results where we examined host proteins stabilized in the presence of a functional Dot/Icm system (those at the extreme low end of ΔPSI values), but we are just as interested in candidates destabilized in the presence of a functional Dot/Icm system (those at the extreme high end of ΔPSI values).



We are also fortunate that the ORFeome v3.1 library is available to us as an arrayed set of ORFs in a Gateway entry vector. Therefore, we will confirm all of our GPS "hits" by picking out ORFs based on graphs where the peak representing the distribution of wild-type *L. pneumophila*-infected cells was clearly in a different bin than the peak representing the distribution of the *ΔdotA*-infected cells (as shown graphically with 5 examples in the figure above). We will then clone each ORF into the original retroviral GPS destination vector described in section 3(c).ii above. These constructs will be individually packaged into retrovirus and then used to transduce HEK 293T cells. Each transduced cell line will be infected with either wild-type *L. pneumophila* or the *dotA* mutant strain. The cells containing the individual ORFs will be then subjected to flow cytometry and changes to EGFP:DsRed ratio will be identified will be compared to the original GPS "hit".

The GPS system is ultimately a screening tool where "hits" must be validated under endogenous promoters and expression levels in cells. We will examine the stability of several candidate "hit" proteins by comparing immunoblots of these host proteins in wild-type *L. pneumophila* and *ΔdotA* infected cells (using commercially available antibodies when available). After identifying and validating candidates, we will have a new "catalog" of host proteins whose stability are altered by *L. pneumophila* Dot/Icm-secreted effectors during infection.

3(c).v. Additional experiments and plans to exploit the information gained from these GPS screens.

1. We strongly believe that GPS can be used to identify host protein targets as well as the protein networks affected by infection with pathogens such as *L. pneumophila*. A key goal for this project is to carefully determine the depth and validity of the data in our screen. The only previously conducted GPS screen (24) identified targets of a ubiquitin ligase, comparing untreated cells with cells expressing a dominant negative inhibitor of that ubiquitin ligase. This screen yielded >350 candidate substrates of the SCF ubiquitin ligase. A screen examining the impact of >150 *L. pneumophila* specific translocated effectors on host protein stability is likely to cause even greater perturbations than the activity of a single ubiquitin ligase. We imagine being able to mine from our data significant pathways in protein turnover that are mediated by infection that have been previously undiscovered. It also should be noted that the ubiquitin ligase GPS screen used the ORFeome v1.1 library (~8,000 ORFS) whereas our use of the ORFeome v3.1 library (12,000 ORFs) should allow even deeper protein stability network analysis. In any new GPS screens, we will employ the just-constructed ORFeome v5.1 library (17,000 ORFs, almost half the human genome) and corresponding microarrays or sequence-based quantitation. Complete coverage of the human genome is a near term goal of the groups assembling the ORFeome libraries. In addition to identifying individual host cell targets, a number of bioinformatics tools are available that will also assist us in revealing regulatory networks of proteins impacted by *L. pneumophila* infection. Together, this information will significantly increase our understanding of *L. pneumophila* infection and the basic cell biology of host-pathogen interactions in general.

2. Little is known about how the majority of *L. pneumophila* Dot/Icm effector proteins are temporally regulated and translocated to host cells, therefore we will conduct identical GPS screens at several other timepoints after infection (besides the 24 h point used above) to gain a more complete understanding of how host cell protein stability changes throughout different stages of *L. pneumophila* infection. By understanding how individual host proteins are altered over the complete *L. pneumophila* replication cycle we can gain insight into *L. pneumophila* growth regulation and work towards interventions that block the progression of early intracellular replication and survival.

3. As we screen, identify, and validate proteins whose stability is altered by *L. pneumophila*-translocated substrates we will also conduct experiments to determine the effect of "preventing"/"counteracting" these individual changes from occurring. For example, with the stabilized proteins where a *L. pneumophila* effector might "increase" the amount of a particular host protein within the cell, we will use siRNA to knock-down that protein within the cell. We then can examine whether this attempt to "counteract" infection-induced

stabilization of a host cell protein makes the cell more resistant to (or perhaps more susceptible to) infection with *L. pneumophila*. For the siRNA experiments, we are fortunate that the groups involved in creating the ORFeome libraries also have produced corresponding siRNA libraries to which we have complete access. In parallel we plan to also overexpress each "destabilized" protein candidate in host cells to see the effect on bacterial replication and development. Again we are fortunate to have access to the arrayed ORFeome libraries in Gateway entry vector. This will greatly facilitate making the mammalian cell overexpression constructs.

Certainly some of the knock-downs or overexpression constructs may be deleterious to the cell or will not be appropriately "tuned" in expression level for us to observe differences in bacterial infectivity or growth. And we certainly expect that with most of our candidates, changing the level of a single protein won't have any effect on infection. But we are optimistic that, either through individual interventions, through "pooled" interventions (such as using multiple RNAis or overexpressing multiple genes), or through insights into regulatory networks during infection, we will be able to use information about changes in host cell protein stability to intervene in the intracellular replication and survival of *L. pneumophila.*

4. As we identify candidate proteins and networks altered during *L. pneumophila* infection, we will use any available small molecule compounds known to affect specific targets – treating cells with the compounds and looking for an effect on *L. pneumophila* replication. Our experiments knocking-down or overexpressing the proteins may also identify host proteins or regulatory pathways that would be exciting to directly target in a chemical screen. Small molecule screening platforms and expertise are available to us at both the Harvard Institute of Chemistry and Cell Biology (http://iccb.med.harvard.edu/) and the Broad Institute of Harvard and MIT (http://www.broadinstitute.org/science/platforms/chemical-biology-platform/chemical-biology-platform).

5. We are confident that our proposed screen examining changes in host protein stability that are dependent on a functional type IV secretion system is the most direct application for the GPS platform with *L. pneumophila*. We have considered conducting a GPS screen that compares changes in protein stability between uninfected and infected cells. However, the scale of this perturbation is so drastic that it would be difficult to tease apart what alterations are actively caused by a *L. pneumophila* infection and which are a response of the cell being infected. While this might be a minor concern in our current screen we can examine individual hits using the *L. pneumophila thyA* mutant strain that has a functional Dot/Icm system (so substrates are secreted initially) but fails to replicate due its thymidine auxotrophy (1, 13). This will allow us to exclude protein alterations that are simply due to *L. pneumophila* intracellular replication and the "stress" caused by the presence of a large vacuole containing replicating bacteria and not from the activity of the Dot/Icm translocation machinery. Furthermore, in normal human cells the TLR pathways would also be activated after *L. pneumophila* infection and proteins in those pathways would certainly be altered. However, HEK 293T do not express the major TLRs therefore limiting the number of hits that result from innate immune responses rather than direct effects of *L. pneumophila* Dot/Icm-secreted substrates. Overall the GPS platform is amenable to many novel screens that explore several different aspects of pathogenesis and protein stability. Certainly many pathogens that affect or manipulate the host cell could also be explored by GPS in this manner and we anticipate the GPS methodology will be broadly applied.

3(c). Approach (Specific Aim 2). Determine the *L. pneumophila* Dot/Icm-translocated substrates that are responsible for alterations in host protein stability.

Over the last decade many investigators have been interested in identifying the function of individual secreted effectors in several different bacterial pathogens. However, with a pathogen like *L. pneumophila* where there are over 150 translocated substrates, many effectors have overlapping functions with other effectors. This has limited progress in determining the role of individual effectors since mutant strains of *L. pneumophila* that are deficient in only one translocated substrate rarely have severe phenotypes (14, 15, 17, 20, 21). Multiple mutations often need to be made in order for an appreciable phenotype to be seen. Furthermore, even in cases where the biochemical activity of a specific effector (e.g. a cysteine protease) is known, determining the host targets remains a daunting task. With our list of host proteins that are altered in stability only when the Dot/Icm system is functional (from Specific Aim 1), we are more strongly equipped to pursue individual effector/host target interactions through gain-of-function analyses that side-step the problem of redundancy.

3(c) vi. Using a gain-of-function screen to identify *L. pneumophila*-translocated effectors that alter the stability of proteins. Since the host proteins we identified in Specific Aim 1 using the GPS platform are stabilized or destabilized only in the presence of Dot/Icm-translocated substrates, we will seek to identify specific *L. pneumophila* effectors that are responsible for these changes. To do this, we will first create stable cell lines transfected with the GPS retroviral construct (described in section 3(c).vi above) where EGFP is fused to specific host proteins whose alterations are essential for *L. pneumophila* infection to progress normally. We will then take these "GPS reporter" cell lines and transfect them individually with the genes encoding the 150 known and putative Dot/Icm-translocated effectors also known as the *L. pneumophila*

"secretome". Craig Roy at Yale University has agreed to provide us with their existing set of the 150 effectors. Once we have the transfected cells, we can use flow cytometry as a high-throughput read-out to determine whether the expression of individual Dot/Icm-translocated effectors recapitulates the shift in the EGFP:DsRed ratio observed when the cells were infected with wild-type *L. pneumophila* compared to the *dotA* mutant. In these experiments, we will avoid possible cytotoxic effects of overexpressing effectors in host cells by using Gateway cloning to shuttle these genes into the destination vector pT-Rex-DEST31 that allows high-level tetracycline-regulated expression of each gene of interest in mammalian cells expressing the Tet repressor. This will allow for temporal regulation of each effector protein and allow us to examine the impact of the various effectors at various time points after expression.

Candidate bacterial effectors that are able to alter stabilization of a specific GPS construct will then be further characterized. Taking advantage of the well-established genetic tools in *L. pneumophila,* we will make clean *in-frame* deletions of these genes. These mutant strains will then be assayed for their ability to alter the specific host protein identified above. If more than one candidate effector is identified for a single host protein, we will make a *L. pneumophila* strain in which all candidate effectors are deleted. Given the functional redundancy among effectors, we may not be able to identify all the effectors involved in altering the stability of a certain host protein. Nevertheless, we believe that this approach will allow us to identify novel bacterial effector/host protein target interactions by focusing on host protein changes that are dependent on Dot/Icm secreted effectors.

3(c) vii. Determining the role of *L. pneumophila* Dot/Icm-translocated substrates that possess E3 ubiquitin ligase domains. As discussed above, redundancy among *L. pneumophila* effectors has made it difficult to determine the specific host function targeted by any particular effector. Effectors have been grouped into "families" whose members share particular functions or functional motifs. Among these families implicated in modulation of host protein stability are the 6 effector proteins carrying domains that are normally associated with E3 ubiquitin ligase activity (3, 11). LegU2/LubX (*Legionella* U-box protein) (2, 11) functions to ubiquitinate the host cell cycle protein Clk1, while Lpp2082, LegU1, LegAU13, LicA and Lpg2160 are associated with multiple components of the host ubiquitination machinery but further functional description has been elusive (3). We will create a *L. pneumophila* strain bearing clean deletions in all six E3 ligase domain effectors. As time allows, we will conduct a new GPS screen comparing this strain to wild-type - allowing us to assess how deletion of a single family of effectors (all E3 ligase domain containing proteins) in an otherwise wild-type *L. pneumophila* alters the stability of the host proteome. Since the GPS platform was developed to identify substrates of mammalian E3 ubiquitin ligases we are confident that this screen will allow us to identify the targets of the E3 ligase domain effectors, and serve as an additional proof-of-principle for the use of GPS. This approach not only will help us dissect how all E3 ligase domain containing effectors in *L. pneumophila* co-opt the eukaryotic ubiquitination machinery to target other host proteins for degradation, but it is another approach that allows us to circumvent functional redundancy with all other "families" of effectors that have similar activities or protein domains.

3(c) viii. Summary. The two aims described here use the highly innovative Global Protein Stability screening platform to identify host proteins that are altered in stability due to the secretion of effectors from the *L. pneumophila* type IV secretion system. Through a directed ORFeome screen as well as a specific gain-of-function study, we will for the first time have an understanding of how secreted bacterial effectors have a global impact on the stability of the human proteome. These approaches open new avenues to identify the specific effectors that are responsible for host protein changes at a level not previously possible. We believe that the ability to catalog how pathogens manipulate host cell proteins at a global scale is likely to bring about a shift in the way host-pathogen interactions are studied in the future.

11. Select Agent Research

No select agents will be used in this project. The BL-2 work proposed in this application have been approved by Harvard's Committee on Microbiological Safety. All safety procedures for use with BL-2 level pathogens will be followed.

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Howard Hughes Medical Institute
Research Laboratories

Stephen J. Elledge, Ph.D.
Investigator

Michael Starnbach, Ph.D.
Department of Microbiology and Molecular Genetics
Harvard Medical School

Oct.12, 2010

Dear Michael:

I'm writing to indicate my support for your application for funding to NIH. I am pleased that we are working together to conduct Global Protein Stability (GPS) screens to identify proteins whose stability is altered during infection with *Legionella pneumophila.* Screening cells infected with microbial pathogens is an application where I feel GPS could make a genuine contribution to our understanding of disease. The members of my lab and I are happy to continue providing advice and access to the libraries for these studies.

Sincerely,

Stephen J. Elledge, Ph.D.

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15. Resource Sharing Plan(s)

Research resources generated with funds from this grant will include DNA constructs and transfected cell lines. These resources, as available, will be freely distributed upon request to qualified academic investigators for non-commercial research. My institution and I will adhere to the NIH Grants Policy on Sharing of Unique Research Resources including the "Sharing of Biomedical Research Resources: Principles and Guidelines for Recipients of NIH Grants and Contracts" issued in December, 1999. http://ott.od.nih.gov/NewPages/Rtguide_final.html. Specifically, material transfers would be made with no more restrictive terms than in the Simple Letter Agreement or the UBMTA and without reach through requirements. Should any intellectual property arise which requires a patent, we would ensure that the technology remains widely available to the research community in accordance with the NIH Principles and Guidelines document.

PHS 398 Checklist

OMB Number: 0925-0001

1. Application Type:

From SF 424 (R&R) Cover Page. The responses provided on the R&R cover page are repeated here for your reference, as you answer the questions that are specific to the PHS398.

* Type of Application:

☒ New ☐ Resubmission ☐ Renewal ☐ Continuation ☐ Revision

Federal Identifier:

2. Change of Investigator / Change of Institution Questions

☐ Change of principal investigator / program director

Name of former principal investigator / program director:

Prefix:
* First Name:
Middle Name:
* Last Name:
Suffix:

☐ Change of Grantee Institution

* Name of former institution:

3. Inventions and Patents (For renewal applications only)

* Inventions and Patents: Yes ☐ No ☐

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

* Previously Reported: Yes ☐ No ☐

4. * Program Income

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)

5. * Disclosure Permission Statement

If this application does not result in an award, is the Government permitted to disclose the title of your proposed project, and the name, address, telephone number and e-mail address of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information (e.g., possible collaborations, investment)?

☐ Yes ☒ No