**PI:** STARNBACH, MICHAEL N  
**Title:** Alteration of host protein stability by Legionella

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<tr>
<td>Michael Starnbach</td>
<td>PD/PI</td>
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**Always follow your funding opportunity's instructions for application format.** Although this application demonstrates good grantsmanship, time has passed since the grantee applied. The sample may not reflect the latest format or rules. NIAID posts new samples periodically: [https://www.niaid.nih.gov/grants-contracts/sample-applications](https://www.niaid.nih.gov/grants-contracts/sample-applications)

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APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

1. **TYPE OF SUBMISSION**
   - Pre-application
   - Application
   - Changed/Corrected Application

2. **DATE SUBMITTED**
   - 10/18/2010
   - 5789461-01-5256928

5. **APPLICANT INFORMATION**
   - 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
   - First Name: Deborah
   - Middle Name: 
   - Last Name: Good
   - Suffix: 
   - Phone Number: 
   - Fax Number: 
   - Email: 

6. **EMPLOYER IDENTIFICATION (EIN) or (TIN):**

7. **TYPE OF APPLICANT:**
   - 0: Private Institution of Higher Education
   - Other (Specify): 
   - Small Business Organization Type
     - Women Owned
     - Socially and Economically Disadvantaged
   - * Is this application being submitted to other agencies? Yes
     - What other Agencies?

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? No

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
    - MA-008

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

14. **PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION**
    - Prefix: Dr.
    - * First Name: Starnbach
    - Middle Name: 
    - Last Name: Michael
    - 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: 
    - Fax Number: 
    - Email: 

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

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

Pre-application
Application
Changed/Corrected Application
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Application
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### 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

Add Attachment  | Delete Attachment  | View Attachment

### 19. Authorized Representative

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<th>Middle Name: A.</th>
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<td>* Last Name: Evans</td>
<td></td>
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<tr>
<td>* Position/Title: Senior Sponsored Research Administrator/Train</td>
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* Signature of Authorized Representative

Evans, Kelly A.

* Date Signed

10/18/2010

### 20. Pre-application

Add Attachment  | Delete Attachment  | View Attachment
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Principal Investigator/Program Director (Last, first, middle): Starnbach, Michael, N.
**President and Fellow of Harvard College**

*Street1: 200 Longwood Avenue
City: Boston
State: MA: Massachusetts
County: Suffolk
Province: USA: UNITED STATES
ZIP / Postal Code: 02115
Project/Performance Site Congressional District: MA-008
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  

8. * Project Narrative  

9. Bibliography & References Cited  

10. Facilities & Other Resources  

11. Equipment  

12. Other 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, FACSscan and FACSaria flow cytometers sorters, oligonucleotide synthesizers, thermal cyclers, HPLC, refrigerators, freezers, -80°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.
### PROFILE - Project Director/Principal Investigator

**Prefix:** Dr.  
**First Name:** Michael  
**Middle Name:** N.  
**Last Name:** Starnbach  
**Suffix:**  
**Position/Title:** Professor  
**Organization Name:** President and Fellows of Harvard University  
**Division:** Harvard Medical School

**Street1:** Harvard Medical School  
**Street2:** 200 Longwood Avenue  
**City:** Boston  
**State:** MA: Massachusetts  
**Country:** USA: UNITED STATES  
**Phone Number:**  
**Fax Number:**  
**E-Mail:**  
**Credential, e.g., agency login:**  

**Project Role:** PD/PI  
**Other Project Role Category:**  
**Degree Type:** Ph.D.  
**Degree Year:** 1992  

**Attach Biographical Sketch**  
**Attach Current & Pending Support**

---

### PROFILE - Senior/Key Person 1

**Prefix:**  
**First Name:**  
**Middle Name:**  
**Last Name:**  
**Suffix:**  
**Position/Title:**  
**Organization Name:**  
**Division:**  

**Street1:**  
**Street2:**  
**City:**  
**State:**  
**Country:** USA: UNITED STATES  
**Phone Number:**  
**Fax Number:**  
**E-Mail:**  

**Credential, e.g., agency login:**  

**Project Role:**  
**Other Project Role Category:**  
**Degree Type:**  
**Degree Year:**  

**Attach Biographical Sketch**  
**Attach Current & Pending Support**
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.**

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<thead>
<tr>
<th>NAME</th>
<th>Michael Starnbach</th>
</tr>
</thead>
<tbody>
<tr>
<td>eRA COMMONS USER NAME</td>
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**EDUCATION/TRAINING** *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

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<th>FIELD OF STUDY</th>
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<tr>
<td>Vassar College, Poughkeepsie, NY</td>
<td>A.B</td>
<td>1987</td>
<td>Biology</td>
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<tr>
<td>Stanford University, Stanford, CA</td>
<td>Ph.D</td>
<td>1992</td>
<td>Micro. &amp; Immuno.</td>
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<tr>
<td>University of Washington, Seattle, WA</td>
<td>None</td>
<td>1992</td>
<td>Postdoctoral Research</td>
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**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).


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

<table>
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<th>* First Name:</th>
<th>Dr.</th>
<th>Michael</th>
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**2. Human Subjects**

Clinical Trial?  
- [ ] No  
- [X] Yes

* Agency-Defined Phase III Clinical Trial?  
- [ ] No  
- [X] Yes

**3. Applicant Organization Contact**

Person to be contacted on matters involving this application

<table>
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<th>Ms.</th>
<th>Deborah</th>
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* Phone Number: 

Fax Number: 

Email: 

* Title:  

Associate Director

* Street1:  

25 Shattuck Street

Street2: 

* City:  

Boston

County/Parish: 

State:  

MA: Massachusetts

* State:  

Province: 

* Country:  

USA: UNITED STATES  

* Zip / Postal Code: 02115-6027
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.
### Budget Period: 1

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

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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

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

Funds Requested ($)

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### Budget Period: 2

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

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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

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

Funds Requested ($)
## PHS 398 Modular Budget, Periods 3 and 4

### Budget Period: 3

**Start Date:**

**End Date:**

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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:**

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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

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**A. Direct Costs**

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* Total Direct Costs

**B. Indirect Costs**

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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 ($)

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**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
   * Section B, Total Indirect Costs for Entire Project Period
   * Section C, Total Direct and Indirect Costs (A+B) for Entire Project Period

2. **Budget Justifications**

   Personnel Justification
   Consortium Justification
   Additional Narrative Justification

---

Principal Investigator/Program Director (Last, first, middle): Starnbach, Michael, N.
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.
# 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.

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### List of Research Plan Attachments

- R21Starnbach2SpecificAimsFeb
- R21Starnbach3ResearchStrategy
- R21StarnbachSelectagentsOct
- R21StarnbachElledgeSupport
- R21Starnbachresourcesharing

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Principal Investigator/Program Director (Last, first, middle): Starnbach, Michael, N.
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 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 over 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 ΔdotA. 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 stability 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 “destabilized” as a result of the *dotA* mutation) to +6 (the theoretical limit of proteins most “stabilized” 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 immunoblot 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
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.


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.
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](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.
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:

- [x] 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:

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- [ ] 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
- [x] 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.

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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
- [x] No