PI: **Raghavan, Rahul**	Title: Elucidating the evolution of Coxiella to uncover critical metabolic pathways	
Received: 06/20/2016	FOA: PA16-200	Council: 01/2017
Competition ID: FORMS-D	FOA Title: Academic Research Enhancement Award (Parent R15)	
1 R15 AI126385-01A1	Dual:	Accession Number: 3949655
IPF: 6297008	Organization: PORTLAND STATE UNIVERSITY	
Former Number:	Department:	
IRG/SRG: ZRG1 IDM-S (81)A	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 1: [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:*
Rahul Raghavan	PORTLAND STATE UNIVERSITY	PD/PI

Additions for Review

Accepted Publication accepted manuscripts

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

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

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

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

1. TYPE OF SUBMISSION*

❍ Pre-application ● Application ❍ Changed/Corrected Application

4.a. Federal Identifier [redacted]

b. Agency Routing Number

2. DATE SUBMITTED | **Application Identifier** | **c. Previous Grants.gov Tracking Number**

5. APPLICANT INFORMATION **Organizational DUNS*:** 0522268000000

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

Person to be contacted on matters involving this application
Prefix: First Name*: Johanna Middle Name: Last Name*: Zimmerman Suffix:
Position/Title: Departmental Research Administrator
Street1*: [redacted]
Street2:
City*: [redacted]
County: [redacted]
State*: [redacted]
Province:
Country*: [redacted]
ZIP / Postal Code*: [redacted]
Phone Number*: 503-[redacted]-2442 Fax Number: Email: [redacted]

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

7. TYPE OF APPLICANT* H: Public/State Controlled Institution of Higher Education

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

8. TYPE OF APPLICATION*
❍ New ● Resubmission
❍ Renewal ❍ Continuation ❍ Revision

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

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

9. NAME OF FEDERAL AGENCY*
National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER
TITLE:

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT*
Elucidating the evolution of Coxiella to uncover critical metabolic pathways

12. PROPOSED PROJECT
Start Date* Ending Date*
04/01/2017 03/31/2020

13. CONGRESSIONAL DISTRICTS OF APPLICANT
OR-003

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

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

Position/Title: Assistant Professor

Organization Name*: PORTLAND STATE UNIVERSITY

Department:

Division:

Street1*: [redacted]

Street2:

City*: [redacted]

County: [redacted]

State*: [redacted]

Province:

Country*: [redacted]

ZIP / Postal Code*: [redacted]

Phone Number*: [redacted] Fax Number: 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 penalties. (U.S. Code, Title 18, Section 1001)

● I agree*

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

18. SFLLL or OTHER EXPLANATORY DOCUMENTATION File Name:

19. AUTHORIZED REPRESENTATIVE

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

Position/Title*: Grants and Agreements Analyst

Organization Name*: Portland State University

Department:

Division:

Street1*: [redacted]

Street2:

City*: [redacted]

County: [redacted]

State*: [redacted]

Province:

Country*: [redacted]

ZIP / Postal Code*: [redacted]

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

Signature of Authorized Representative*	Date Signed*
Karena Bayruns	06/20/2016

20. PRE-APPLICATION File Name:

21. COVER LETTER ATTACHMENT File Name:CoverLetter-R15-2016-resub.pdf

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

Project/Performance Site Location(s)

Project/Performance Site Primary Location

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

Organization Name:	Portland State University
Duns Number:	[redacted]
Street1*:	[redacted]
Street2:	
City*:	[redacted]
County:	[redacted]
State*:	[redacted]
Province:	
Country*:	[redacted]
Zip / Postal Code*:	[redacted]
Project/Performance Site Congressional District*:	OR-003

File Name

Additional Location(s)

Tracking Number: GRANT12198450
Funding Opportunity Number: PA-16-200. Received Date: 2016-06-20T20:28:55.000-04:00

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

RESEARCH & RELATED Other Project Information

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

1.a. If YES to Human Subjects

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

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

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

IRB Approval Date:

Human Subject Assurance Number

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

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ❍ Yes ❍ No

IACUC Approval Date:

Animal Welfare Assurance Number

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

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

4.b. If yes, please explain:

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

4.d. If yes, please explain:

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

5.a. If yes, please explain:

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

6.a. If yes, identify countries:

6.b. Optional Explanation:

	Filename
7. Project Summary/Abstract*	ProjectSummary-R15-2016-resub.pdf
8. Project Narrative*	Relevance-to-public-health-R15-2016-resub.pdf
9. Bibliography & References Cited	References-R15-2016-resub-v5.pdf
10.Facilities & Other Resources	Facilities-resources-R15-2016-resub.pdf
11.Equipment	Major-equipment-R15-2016-resub-final.pdf

Tracking Number: GRANT12198450

Funding Opportunity Number: PA-16-200. Received Date: 2016-06-20T20:28:55.000-04:00

PROJECT SUMMARY/ABSTRACT

Coxiella burnetii's pathogenicity depends on its ability to grow in a lysosome-derived hostile vacuole within human cells. However, metabolic processes critical to *C. burnetii*'s intracellular growth are largely unknown. This lack of knowledge has prevented both the understanding of its basic biology and pathogenesis, and the development of better therapeutic agents. The *long-term goal* is to understand the molecular details of *Coxiella*'s distinctive physiology, and to apply this knowledge to developing novel therapeutic strategies. The *objective* of this application is to identify metabolic pathways that are vital to *C. burnetii*'s intracellular growth. The *central hypothesis*, which was formulated based on preliminary data, is that *C. burnetii* evolved from a tick-associated ancestor by acquiring critical metabolic genes through horizontal gene transfer (HGT). A novel evolutionary genomics approach will be used to identify metabolic pathways that are critical to *C. burnetii*'s intracellular growth. The *rationale* for the proposed research is that once metabolic processes important to *C. burnetii*'s intracellular growth are identified, pharmacological agents that block these pathways could be developed to treat chronic infections more effectively. The objective of this project will be accomplished by three *specific aims*: **(1)** Identify metabolic pathways that distinguish *C. burnetii* from tick-associated *Coxiella*. The working hypothesis is that genes critical to *C. burnetii*'s intracellular physiology will not be present in avirulent tick-associated *Coxiella*. The genome of a closely related *Coxiella* from the tick *Ornithodoros rostratus* will be sequenced and compared to *C. burnetii*'s genome. **(2)** Define metabolic pathways that are critical to *C. burnetii*'s intracellular growth. The working hypothesis is that genes acquired via HGT are being maintained in *C. burnetii* because they are critical to the pathogen's physiology. Phylogenetic approaches will be used to identify HGT-derived genes, and their functions will be validated using RNA-seq and genetic tools. **(3)** As a proof of principle, determine the importance of heme biosynthesis to *C. burnetii's* intracellular growth. The working hypothesis is that heme biosynthesis is crucial to *Coxiella*'s growth. Heme production and intracellular growth of heme pathway-deficient strains will be assayed. This study is *innovative* because it (a) uses a novel approach that overcomes the current limitations in studying *Coxiella* at a genome-wide scale, and (b) is based on a novel concept that the human pathogen evolved from a tick symbiont via massive HGT. The proposed project is *significant* because it will (a) uncover metabolic pathways that are critical to the pathogen's intracellular growth, (b) identify new therapeutic targets, for example, HemA and HemL are essential for heme biosynthesis in *C. burnetii* but are not present in humans cells, (c) provide a model approach for identifying genes involved in host adaptation, which could be applied broadly to other pathogens such as *Francisella* and *Rickettsia* spp. where avirulent tick symbionts could be compared to virulent human-specialized strains.

Relevance to public health

Coxiella burnetii is a zoonotic bacterium that causes Q fever and chronic endocarditis. Metabolic processes critical to its intracellular growth and pathogenesis are not known. The proposed project will identify important metabolic pathways in *C. burnetii* using a novel evolutionary genomics approach that will overcome the current technical difficulties in studying *Coxiella* at a genome-wide scale. The application will also provide a useful model for identifying key metabolic processes in other human pathogens and will identify new genes that could be targeted to treat chronic Q fever more effectively.

FACILITIES AND OTHER RESOURCES

Laboratory:
The PI's main laboratory is located in the Collaborative Life Sciences building (CLSB), a new academic and research facility shared between Portland State University (PSU), Oregon Health and Science University (OHSU), and Oregon State University (OSU). The main lab is ~900 sq. ft. with four lab benches and is equipped to perform all proposed molecular biology experiments. The lab is part of an open floor plan and is flanked by labs from OHSU and OSU, which foster natural collaborations. A separate BSL-2 certified ~200 sq. ft. room is equipped with biosafety hoods, CO2 incubators, inverted microscope etc. to perform bacterial culture and host cell infection assays. Two walk-in cold rooms, a shared equipment room, a microscopy room, and a sterilization room equipped with autoclave and dishwasher are located immediately adjacent to the main lab.

Animal: None

Clinical: None

Computer:
The PI's laboratory is equipped with two Linux servers (24 core with 256 GB RAM; 12 core with 128 GB RAM), one MacPro (64 GB RAM, 2 TB hard drive), two iMac computers, one MacBook Pro, several PCs, and a 10 TB RAID storage array. We also have access to three university-wide research servers (12 core with 96 GB RAM; 16 core with 126 GB RAM; 28 core with 250 GB RAM) and a 23-node, 184-core parallel-processing Linux cluster. In addition, there is a 20-terminal computer lab in the building. The whole building has wireless and hard-wired access to the local network and to the Internet. Two full-time IT technicians serve the Biology department.

Office:
The PI has a ~150 sq. ft. office with a MacBook Pro computer. In addition, there is a second ~150 sq. ft. office available for postdocs and cubicles with data and phone connections for graduate students.

Other:
The research in the PI's lab benefits from close collaborations with OHSU and OSU faculty members that occupy the shared building (CLSB) where the PI's lab is located. In addition, the PI's lab maintains regular contact by sharing reagents, performing collaborative experiments, and conducting combined lab meetings with several research groups at the OHSU main campus located within a 5-minute tram ride from CLSB. These research groups include Dr. Richard Goodman's lab that work on eukaryotic microRNA, Dr. Scott Landfear's group that work with the eukaryotic pathogen *Leishmania*, Dr. Eric Cambronne's group that works on the pathogenesis of *Legionella pneumophila*, and Dr. Justin Merritt's lab that works on the genetics of the oral pathogen *Streptococcus mutans*. In addition, the PI has access to all OHSU core facilities (Proteomics, Genomics, Sanger sequencing, Microscopy etc.).

A profile of the applicant's institution:
Portland State University is a public state urban university located in downtown Portland, Oregon. It is a relatively young school (founded in 1946), but is one of the largest universities in Oregon. A combination of targeted outreach programs and proximity to the state's population and industry results in an unusually large number of first generation college students attending PSU. In 2014, approximately 30,000 students were enrolled in PSU and 4300 bachelor's degrees and 1700 graduate degrees were awarded. PSU places more students into medical schools than any other university in Oregon, and a large portion of our students have gone on to pursue successful research careers.

The Department of Biology has approximately 1000 undergraduate majors and about 70 graduate students. Student research is highly encouraged in the department and each PI's lab typically has three to four undergraduate student research trainees at any given time. Undergraduate research training at PSU is fostered by regular, one-on-one interactions between students and the PI. Several mechanisms are in place

at the Biology department to foster research activity by students, including the Biology Honors program, the Forbes Lea Endowed Fund for Student Research, and The David Clark Endowment Fund that supports student travel to scientific meetings. Additionally, students are exposed to cutting edge science on a daily basis because most biology lectures and labs are held at CLSB, which houses the OHSU Dental School, parts of OHSU Medical School, OSU College of Pharmacy, and 30 research labs from OHSU, OSU and PSU.

Impact of an AREA award on student research:

PSU is not a research-intensive university when compared to major academic research institutions in the United States. However, PSU is highly committed to growing its research infrastructure. R&D expenditures continue to grow, with an average annual growth rate over the past 10 years of 10.6%. PSU's Science Building has recently undergone a major ($45.3 million) renovation and now has improved research and teaching labs. Recently PSU in collaboration with OHSU and OSU set up the Collaborative Life Sciences building (CLSB), a unique $295 million project that combines the resources of three major universities to offer the best possible science education and research opportunities for students. Biology and Chemistry teaching labs and four PSU research groups (including the PI's lab) are now located at CLSB. The Biology department has a robust research program. Currently there are 29 externally supported projects (two in PI's lab) among the 20 faculty members. NSF supports most of these projects and NIH funds five. The PI is highly committed to student research. The PI has mentored and published with several undergraduate students during his graduate and postdoctoral tenures (list provided in biosketch). The PI currently has three undergraduate research trainees in the lab and four graduate students. In addition to his research program, the PI teaches courses in Microbiology (BI480), and Bioinformatics (BI410/510) that offer the opportunity to introduce biomedical research to undergraduates, and to attract students to partake in research experience. He therefore has an opportunity to significantly impact the number of graduates at his institution that go on to pursue health-related careers. AREA awards are critical to this mission.
The work proposed here has been designed and structured to provide research opportunities to several students over the three-year life of the grant. **Jessica Millar**, a current graduate student in the lab generated most of the preliminary bioinformatics data for this project while she was an undergraduate researcher. **Abraham Moses** started as an undergraduate researcher in the lab and now is a Research Associate in the lab. He is adept at *Coxiella* culture and helped to generate the experimental portion of the preliminary data. Currently he is working with **Todd Hinsch**, an undergraduate researcher to construct pJC-CAT vectors to delete *Coxiella* small RNA genes. **Jonathan Gerhart**, who is a first year graduate student in the lab, is working closely with **Melissa Holler** an undergraduate researcher to sequence the genomes of endosymbionts present in various ticks.

Institutional investment in the success of the Early Stage Investigator:

The Chair of Biology Department will provide a modest teaching load to the PI to facilitate the research outlined in the proposal (please see Letter of Support from the Chair of Biology Department). The PI has been assured of continued assignment of space and will be assigned more space if required. The PI has full access to a variety of shared equipment, research facilities and cores across PSU and OHSU campuses. In addition to providing the PI with a substantial startup package, PSU has supported the PI by providing a Research Stimulus Grant and a Faculty Enhancement Grant, and the Biology department supports graduate students in PI's lab through graduate assistantships. Additionally, The Medical Research Foundation at OHSU has also supported the PI's research by providing a New Investigator Grant. Moreover, the PI's lab is part of a consortium of 30 research groups from OHSU, OSU and PSU that collaborate extensively by sharing equipment and reagents, and by holding regular meeting and social events.

MAJOR EQUIPMENT:

Items within PI's laboratory:
Eppendorf gradient thermal cycler, ABI thermal cycler, Agilent real-time thermal cycler, New Brunswick Tri-gas incubator, New Brunswick CO2 incubator, bacterial incubators, shaking incubators, Perkin-Elmer Victor Multilabel plate reader, Bio-Rad spectrophotometer, BioSpec beadbeater, refrigerators, –20°C freezers, –80°C freezer, Eppendorf thermomixer, centrifuges, shaking platforms, gel electrophoresis equipment, Gel documentation system, Thermo Scientific Biosafety hoods, NanoDrop, Leica DM750 phase contrast microscope, Leica DMIL inverted microscope, Leica TCS SPE Confocal microscope, high performance computers.

Common items available to PI:
Agilent Bio-analyzer, Ultracentrifuges, Li-Cor Odyssey infrared imaging system, Covaris S2 ultrasonicator, MALDI-TOF Mass spec, Shimadzu HPLC, scanning electron microscope, transmission electron microscope, fluorescent microscope, Ion Proton sequencer, cold rooms, high performance computer network and servers.

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

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

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

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

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

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

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

Degree Type: PhD Degree Year: 2008

	File Name
Attach Biographical Sketch*:	Biosketch-R15-2016-resub.pdf
Attach Current & Pending Support:	

Tracking Number: GRANT12198450
Funding Opportunity Number: PA-16-200 . Received Date: 2016-06-20T20:28:55.000-04:00

BIOGRAPHICAL SKETCH

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

NAME: Rahul Raghavan

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

POSITION TITLE: Assistant Professor of Biology

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of Calicut, India	BSc	08/1995	Biology and Chemistry
Manipal University, India	MSc	08/1998	Medical Microbiology
University of Montana, Missoula, MT	PhD	08/2008	Molecular Microbiology
Yale University, New Haven, CT	Postdoc	08/2012	Microbial Genomics

A. PERSONAL STATEMENT

Even after seven decades of research, most aspects about the origin, basic biology, and pathogenesis of *Coxiella burnetii* remain unknown. One of the biggest gaps is in our understanding of the pathogen's unique physiology, which is critical to its pathogenicity. I propose to use a novel evolutionary-genomic approach to uncover *C. burnetii*'s evolutionary history, and use this knowledge to define metabolic genes critical to its intracellular growth and virulence. The outcomes of this project will positively impact the field by providing a set of metabolic proteins that could be targeted to develop novel therapeutic agents. I am ideally prepared to undertake this project because of my unique combination of expertise in molecular microbiology, bioinformatics, high-throughput sequencing, phylogenetics and genomics. I have also setup ongoing collaborations with establish researchers to ensure the success of the proposed project (see Letters of Support).

As a doctoral student in Dr. Mike Minnick's lab (2004-2008), I got trained in working with *Coxiella* by characterizing horizontally acquired selfish genetic elements. I discovered that *C. burnetii*'s growth is likely regulated by introns (ribozymes) through translational inhibition, and showed that by blocking intron splicing using Pentamidine, growth of the bacterium could be significantly reduced, thus identifying a novel therapeutic strategy. In 2009, I moved to Dr. Howard Ochman's lab as a postdoc where I was trained in bioinformatics, high-throughput sequencing and genome evolution. In my new lab at PSU (since Fall 2012), I have applied my knowledge in pathogenomics to explore entirely new areas of *Coxiella* research: (1) In collaboration with Dr. Minnick, we identified the first set of non-coding RNAs in *C. burnetii*; (2) in collaboration with Drs. Eric Cambronne and Scott Landfear at OHSU, we described the first set of microRNAs produced by human macrophages in response to *C. burnetii* infection; (3) in collaboration with Dr. Joseph Gillespie at University of Maryland, we sequenced the first genome of a non-pathogenic *Coxiella*. In total, I have published 14 peer-reviewed articles on *Coxiella*, and have nine publications on various aspects of bacterial genome analyses.

An AREA grant will provide me the opportunity to build on my initial successes to set up a nationally recognized research program. Although PSU is not a research-intensive university, I have the full support of my department and college to pursue cutting edge research. Moreover, my research group is part of a highly interactive and productive research environment where scientists from PSU, Oregon Health and Sciences University, and Oregon State University conduct collaborative research. The AREA grant will also help support student research in my lab. I currently have three undergraduate researchers in my lab. In the three years at PSU, I have mentored 15 undergraduate students, including two students who performed their Honors theses in my lab. I also mentor four graduate students at various stages of their career (a student who graduated this year, a 2nd year student, and two 1st year students). Two of my graduate students, our lab manager, our

Bioinformatician, and IT manager started out as undergraduate researchers in my lab. This organic approach has been very fruitful because all of them are very productive; e.g., my senior grad student (Fenil) has three publications; my second year student (Jess), who received NSF GRFP, has two publications with another under review, and another in development, and a first year student (Jon) has already submitted his first first-author paper. In addition, several undergraduate students who worked in my lab were able to find employment as research technicians at OHSU and other research centers in Portland. I also encourage students to attend local and national conferences (one student received an ASM travel award). I meet with each student regularly and the whole lab meets weekly to discuss latest research or new journal articles. Once a month we conduct a combined lab meeting with two other labs at OHSU (Dr. Justin Merritt's and Dr. Eric Cambronne's labs), providing the students with a broad perspective on microbial research. Some of my recent *Coxiella* papers:

1. **Raghavan R.** 2016. A repeat motif on a *Coxiella* effector protein facilitates apoptosis inhibition. *Virulence* 7:369-371.
2. Millar JA, Valdes R, Kacharia FR, Landfear SM, Cambronne ED, **Raghavan R**. 2015. *Coxiella burnetii* and *Leishmania mexicana* residing within similar parasitophorous vacuoles elicit disparate host responses. *Front Microbiol* 6:794.
3. Smith TA, Driscoll T, Gillespie JJ, **Raghavan R**. 2015. A *Coxiella*-like endosymbiont is a potential vitamin source for the Lone Star Tick. *Genome Biol Evol* 7:831-838.
4. Warrier I, Hicks LD, Battisti JM, **Raghavan R**, Minnick MF. 2014. Identification of Novel small RNAs and characterization of the 6S RNA of *Coxiella burnetii*. *PLoS ONE* 9:e100147.

B. POSITIONS AND HONORS

Positions and Employment

1998-02	Lecturer, School of Health Sciences, University of Calicut, India
2002-04	Research Scientist, St John's Research Institute, Bangalore, India
2004-08	Graduate Assistant. Division of Biological Sciences, University of Montana
2008-09	Postdoctoral Fellow, Division of Biological Sciences, University of Montana
2009-12	Postdoctoral Associate, Yale University (Started at University of Arizona)
2012-present	Assistant Professor, Department of Biology, Portland State University

Teaching Awards

2005	Outstanding Teaching Assistant. Division of Biological Sciences, University of Montana
2015	Commendation for excellent teaching from Portland State University College of Liberal Arts and Sciences.

Other Awards and Honors

2006	Travel Award from the American Society for Rickettsiology.
2007	Co-chaired the scientific session on Genetics, Physiology and Metabolism at the 21st Annual Meeting of the American Society for Rickettsiology. Colorado Springs, CO.
2016	Travel Award from the American Society for Rickettsiology.

Professional Membership

American Society for Microbiology
American Society for Rickettsiology
Society for Molecular Biology and Evolution
American Heart Association

Students mentored

During PhD

Linda Hicks	(Undergraduate, University of Montana, Missoula, MT): 2005.

During Postdoc

Ellen DeGennaro	(Undergraduate, Vassar College, Poughkeepsie, NY): 2010.
Alan Sage	(Undergraduate, Yale University, New Haven, CT): 2010.
Cindy Barlan	(MS student, Quinnipiac University, Hamden, CT): 2011.

Daniel Phillips (MS student, University of New Haven, West Haven, CT): 2011.

As PI (current students underlined)

Christine Sislak (MS student): 2012-2013.
Niveditha Handral (Undergraduate): 2012.
Jess Millar (Undergraduate, later joined the lab as graduate student): 2012-2016.
Brian Wendel (Graduate student rotation): 2013.
Abraham Moses (Undergraduate, later joined the lab as Research Associate): 2012-2016.
Katharine Saucier (Undergraduate): 2012-2014.
Todd Smith (Undergraduate, later joined the lab as Bioinformatician): 2012-2015.
Savannah Smart (Undergraduate): 2013-2014.
Fenil Kacharia (Undergraduate, later joined the lab as graduate student): 2013-2016.
Colleen Campbell (Undergraduate): 2013-2014.
Ashleigh Mustain (Undergraduate): 2013-2014.
Ali Hourmanish (Undergraduate): 2013-2014.
Rachel Champaigne (Honors student): 2013-2015
Robyn Reid (Honors student): 2013-2015
Melissa Hollar (Undergraduate): 2014-2016
Todd Hinsch (Undergraduate): 2014-2016
Jim Archuleta (Undergraduate, also works as the lab IT manager: 2015-2016)
Jonathan Gerhart (Graduate student): 2015-2016
Austin Wright (Graduate student): 2015-2016

Selected Presentations at Professional Meetings (students underlined)

1. Moses AS, Millar JA, Bonazzi M, Beare PA, Raghavan R. 2016. Horizontally acquired tRNA and metabolic genes fortify *Coxiella burnetii*'s physiology. National Meeting of the American society for Rickettsiology. Big Sky, MT.
2. Millar JA, Raghavan R. 2016. A sewage microbiome is dominated by *Arcobacter cryaerophilus* that expresses multiple drug resistance and virulence genes. ASM Microbe, Boston, MA.
3. Gerhart JG, Raghavan. 2016. A *Francisella*-like endosymbiont in the Gulf Coast tick evolved from a mammalian pathogen. Entomological Society of America North Central Meeting. Cleveland, OH.
4. Kacharia F, Merritt J, Raghavan R. 2015. Evolution of regulatory RNAs from vestigial bacteriophage genes. Regulating with RNA in Bacteria and Archaea Conference. Cancun, Mexico.
5. Kacharia F, Raghavan R. 2015. Quantifying evolution to gauge function. Annual Meeting of the Pacific Northwest Quantitative Biology Group. Portland, OR.
6. Smith TA, Raghavan R. 2015. A *Coxiella*-like endosymbiont is a potential vitamin source for the Lone Star Tick. National Meeting of the American society for Rickettsiology, Lake Tahoe, CA.
7. Millar JA, Raghavan R. 2015. *Coxiella burnetii* and *Leishmania mexicana* residing within similar parasitophorous vacuoles elicit disparate host responses. General Meeting of the American Society for Microbiology. New Orleans, LA.
8. Millar JA, Raghavan R. 2014. A horizontally acquired tRNA facilitates *Coxiella burnetii* adaptation to an extreme environment. General Meeting of the American Society for Microbiology. Boston, MA.

C. CONTRIBUTION TO SCIENCE

1. Genome sequence of an avirulent *Coxiella* present in the Lone Star tick. We recently sequenced the complete genome of a bacterium related to *Coxiella burnetii* from the Lone star tick *Amblyomma americanum*. We showed that the tick-associated *Coxiella* does not have any virulence factors but could compensate for the lack of vitamins and cofactors in the tick's blood meal. This bacterium is the closest known relative of *C. burnetii* with a fully sequenced genome, thereby facilitating new insights into the evolution of this pathogen. Our results are also important for strategies geared towards controlling *A. americanum* and the pathogens it vectors. The lead author in this study was Todd Smith, an undergraduate student in my lab. Jonathan Gerhart, a new graduate student in lab is currently assembling the genome of a *Francisella* endosymbiont present in the related Gulf Coast tick *Amblyomma maculatum* (manuscript is currently under review). He will sequence and assemble the genome of a *Coxiella* present in the soft-tick *Ornithodoros rostratus* as part of Specific Aim 1.

Smith TA, Driscoll T, Gillespie JJ, **Raghavan R**. 2015. A *Coxiella*-like endosymbiont is a potential vitamin source for the Lone Star Tick. *Genome Biol Evol* 7:831-838.

2. Genome-wide analyses of human macrophage response to *C. burnetii* infection. *Coxiella* and *Leishmania* (eukaryote) are the only pathogens that thrive within an acidic lysosome-derived vacuole inside human macrophages. To begin to understand the parallel evolution of this unique intracellular niche, Jessica Millar, a graduate student in my lab infected human macrophages with either pathogen and identified genes, mRNA-isoforms and microRNAs (miRNAs) that were differentially expressed between infected and uninfected cells. We identified apoptosis-related miRNAs as major regulators during infection, and intriguingly detected several isoforms of mRNAs that were specifically expressed during infection. This project was carried out in collaboration with Prof. Scott Landfear and Dr. Eric Cambronne at OHSU. We are now following up on these clues to identify the molecular mechanisms that promote infections by these intracellular extremophiles. Interactions between pathogens and host cells at the RNA level is an underexplored area of research. Through this project we have now expanded our capability to study ncRNAs and mRNAs in human cells.

Millar JA, Valdes R, Kacharia FR, Landfear SM, Cambronne ED, **Raghavan R**. 2015. *Coxiella burnetii* and *Leishmania mexicana* residing within similar parasitophorous vacuoles elicit disparate host responses. *Front Microbiol* 6:794.

3. Identification of horizontally derived genetic elements in *C. burnetii* and novel use of a pharmacological agent. As part of my doctoral dissertation I studied non-coding RNAs in *Coxiella*. This was an entirely new venture in my advisor's lab, which I spearheaded because of my interest in understanding the evolution and possible functions of RNA-based selfish elements. I discovered a novel Group I intron that uses different cofactors for each splicing step, and described two other selfish genetic elements – an intervening sequence and an intein – in this pathogen. The introns have to splice out of the rRNAs in order for functional ribosomes to form. Exploiting this Achilles' heel, I used Pentamidine, a splice inhibitor to block *Coxiella*'s growth and showed that this is a good strategy to develop novel anti-*Coxiella* therapeutic agents. In these studies I mentored Linda Hicks, an undergraduate student.

Raghavan R, Miller SR, Hicks LD, Minnick MF. 2007. The unusual 23S rRNA gene of *Coxiella burnetii*: two self-splicing group I introns flank a 34-base-pair exon and one element lacks the canonical omega G. *J Bacteriol* 189:6572-6579.

Raghavan R, Hicks LD, Minnick MF. 2008. Toxic introns and parasitic intein in *Coxiella burnetii*: legacies of a promiscuous past. *J Bacteriol* 190:5934-5943.

Raghavan R, Hicks LD, Minnick MF. 2009. A unique group I intron in *Coxiella burnetii* is a natural splice mutant. *J Bacteriol* 191:4044-4046.

Minnick MF, Hicks LD, Battisti JM, **Raghavan R**. 2010. Pentamidine inhibits *Coxiella burnetii* 23S rRNA intron splicing and intracellular growth. Int J Antimicrob Agents 36: 380-382.

4. Genome-wide identification and characterization of non-coding RNAs in *Coxiella*, *E. coli*, and *Salmonella*. During my postdoc, I developed an RNA-seq-based approach to identify novel ncRNAs in bacteria. This approach allowed us to discover several novel ncRNAs in *E. coli*, a well-studied model organism. On this project, I independently worked out most of the technical details of ncRNA discovery because these were completely new areas of research for both my postdoc adviser (Dr. Ochman), and our collaborator Dr. Groisman. In addition, I mentored Alan Sage, an undergraduate student. Later, while I was setting up my lab at PSU, I used the same approach to identify novel ncRNAs in *Coxiella* in collaboration with Dr. Minnick. I performed all the RNA-seq and bioinformatics analyses for this project, and identified all the ncRNAs. This was the first paper to describe ncRNAs in *Coxiella*. As a project that bridged my postdoc and faculty tenures, my students (Fenil Kacharia, Jessica Millar, and Christine Sislak) and I published one of the first clear examples of sRNA evolution in bacteria. I conceived this study, and guided my students to perform experiments that showed that bacterial sRNAs are created and destroyed by genome rearrangements.

Raghavan R, Groisman EA, Ochman H. 2011a. Genome-wide detection of novel regulatory RNAs in *E. coli*. *Genome Res* 21:1487-1497.

Raghavan R, Sage A, Ochman H. 2011b. Genome-wide detection of transcription start sites yield a novel thermo-sensing RNA and new CRP-regulated genes in *E. coli*. *J Bacteriol* 193:2871-2874.

Warrier I, Hicks LD, Battisti JM, **Raghavan R**, Minnick MF. 2014. Identification of Novel small RNAs and characterization of the 6S RNA of *Coxiella burnetii*. *PLoS ONE* 9:e100147.

Raghavan R, Kacharia FR, Millar JA, Sislak CD, Ochman H. 2015. Genome rearrangements can make and break small RNA genes. *Genome Biol Evol* 7:557-566.

5. Evolution of antisense RNAs and bacterial nucleotide composition. The discovery of genome-wide production of antisense RNA has upended our traditional understanding of bacterial transcription. I used a novel strand-specific sequencing approach to investigate the transcriptomes of *E. coli* and *Salmonella*. This paper has become a benchmark in the field because it showed for the first time that although antisense transcription is pervasive, most individual asRNA are not conserved, indicating that most antisense RNAs are likely non-functional. Other labs have extended our study to several other bacteria.

Understanding the significance of the broad range of base compositions (13% to 75% G+C) observed in bacteria has been a vexing problem in microbiology. For over 50 years it has been assumed that selection has no role in determining genomic G+C content. However, we reasoned that because 90% of bacterial genomes are composed of protein-coding genes, any small selection occurring on the base composition of all mRNAs would influence the genomic G+C content. Using *E. coli* expressing the same protein encoded by mRNAs with varying G+C%, I showed that differences in mRNA base compositions affected bacterial fitness. This study, for the first time, revealed a selective force that favors high G+C ratio in bacterial genes.

Raghavan R, Sloan DB, Ochman H. 2012. Antisense transcription is pervasive but rarely conserved in enteric bacteria. *mBio* 3:e00156-12.

Raghavan R, Kelkar YD, Ochman H. 2012. A selective force favoring increased G+C content in bacterial genes. *Proc Natl Acad Sci USA* 109:14504-14507.

I have published 23 peer-reviewed articles till date. My complete list of publications is available at:

http://www.ncbi.nlm.nih.gov/myncbi/browse/collection/41517339/?sort=date&direction=descending

D. RESEARCH SUPPORT

Ongoing Research Support

1. NIH Subaward (11/01/2015-08/31/2016)
   This project will analyze the transcriptomes of *Streptococcus mutans*.
   Role: Collaborator (PI: Justin Merritt)

[redacted]

4. Portland State University Faculty Enhancement Grant (07/01/2014-06/30/2016)
   This project will examine nucleotide modifications in bacterial sRNAs.
   Role: PI
5. Portland State University Startup Fund (09/01/2012-06/30/2017)
   This fund helped me setup my new lab and allowed me to generate data and publish papers.
   Role: PI

Completed Research Support

[redacted]

2. Portland State University Research Stimulus Grant (04/01/2013–03/31/2014)
   This grant helped me establish collaborations with research groups at OHSU, and to generate preliminary data to apply for NIH grants.
   Role: PI

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

RESEARCH & RELATED BUDGET - SECTION A & B, Budget Period 1

ORGANIZATIONAL DUNS*: [redacted]

Budget Type*: ● Project ❍ Subaward/Consortium

Enter name of Organization: PORTLAND STATE UNIVERSITY

Start Date*: 04-01-2017 **End Date*:** 03-31-2020 **Budget Period:** 1

A. Senior/Key Person

	Prefix	First Name*	Middle Name	Last Name*	Suffix	Project Role*	Base Salary ($)	Calendar Months	Academic Months	Summer Months	Requested Salary ($)*	Fringe Benefits ($)*	Funds Requested ($)*
1 .		Rahul		Raghavan		PD/PI	[redacted]			9.0	[redacted]	[redacted]	[redacted]

Total Funds Requested for all Senior Key Persons in the attached file

Additional Senior Key Persons: File Name:

Total Senior/Key Person [redacted]

B. Other Personnel

Number of Personnel*	Project Role*	Calendar Months	Academic Months	Summer Months	Requested Salary ($)*	Fringe Benefits*	Funds Requested ($)*
	Post Doctoral Associates						
2	Graduate Students			[redacted]	[redacted]	[redacted]	[redacted]
2	Undergraduate Students			[redacted]	[redacted]	[redacted]	[redacted]
	Secretarial/Clerical						
1	Research Associate	14.4			[redacted]	[redacted]	[redacted]
5	**Total Number Other Personnel**					**Total Other Personnel**	[redacted]

Total Salary, Wages and Fringe Benefits (A+B) [redacted]

RESEARCH & RELATED Budget {A-B} (Funds Requested)

Tracking Number: GRANT12198450
Funding Opportunity Number: PA-16-200 . Received Date: 2016-06-20T20:28:55.000-04:00

RESEARCH & RELATED BUDGET - SECTION C, D, & E, Budget Period 1

ORGANIZATIONAL DUNS*: [redacted]

Budget Type*: ● Project ❍ Subaward/Consortium

Organization: PORTLAND STATE UNIVERSITY

Start Date*: 04-01-2017 **End Date*:** 03-31-2020 **Budget Period:** 1

C. Equipment Description	
List items and dollar amount for each item exceeding $5,000	
Equipment Item	**Funds Requested ($)***
Total funds requested for all equipment listed in the attached file	
Total Equipment	**0.00**
Additional Equipment: File Name:	

D. Travel	**Funds Requested ($)***
1. Domestic Travel Costs (Incl. Canada, Mexico, and U.S. Possessions)	[redacted]
2. Foreign Travel Costs	
Total Travel Cost	[redacted]

E. Participant/Trainee Support Costs		**Funds Requested ($)***
1. Tuition/Fees/Health Insurance		
2. Stipends		
3. Travel		
4. Subsistence		
5. Other:		
Number of Participants/Trainees	**Total Participant Trainee Support Costs**	**0.00**

RESEARCH & RELATED Budget {C-E} (Funds Requested)

Tracking Number: GRANT12198450

Funding Opportunity Number: PA-16-200 . Received Date: 2016-06-20T20:28:55.000-04:00

RESEARCH & RELATED BUDGET - SECTIONS F-K, Budget Period 1

ORGANIZATIONAL DUNS*: [redacted]

Budget Type*: ● Project ❍ Subaward/Consortium

Organization: PORTLAND STATE UNIVERSITY

Start Date*: 04-01-2017 **End Date*:** 03-31-2020 **Budget Period:** 1

F. Other Direct Costs	Funds Requested ($)*
1. Materials and Supplies	[redacted]
2. Publication Costs	[redacted]
3. Consultant Services	
4. ADP/Computer Services	
5. Subawards/Consortium/Contractual Costs	
6. Equipment or Facility Rental/User Fees	
7. Alterations and Renovations	
Total Other Direct Costs	[redacted]

G. Direct Costs	Funds Requested ($)*
Total Direct Costs (A thru F)	[redacted]

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)*
1 . MTDC	48.5	[redacted]	[redacted]
		Total Indirect Costs	[redacted]

Cognizant Federal Agency DHHS, Jeannette Lu, 415-556-1704
(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs	Funds Requested ($)*
Total Direct and Indirect Institutional Costs (G + H)	[redacted]

J. Fee	Funds Requested ($)*

K. Budget Justification*	File Name: R15-BudgetJustification-june2016-v1.pdf (Only attach one file.)

RESEARCH & RELATED Budget {F-K} (Funds Requested)

Budget Justification

PERSONNEL

Principal Investigator

Rahul Raghavan, PhD**,** will commit 3 summer months of effort to this project each year. He will be responsible for the overall administration and direction of the project. He will be actively involved in planning experimental approaches, mentoring graduate and undergraduate students, interpretation of results, preparation of manuscripts, and dissemination of findings.

Research Associate

Abraham Moses, BS, is requesting 4.80 calendar months of salary support each year (0.40 FTE) to work on the project. He will concentrate on elucidating the role of heme biosynthesis as described in the proposal (*Specific Aim 3*). He is highly competent in all aspects of the proposed research including tissue culture, *Coxiella* culture and molecular techniques. He generated the preliminary data pertaining to the importance of heme biosynthesis to *Coxiella*'s intracellular growth.

Graduate Research Assistants

Two graduate students will each work 0.99 summer months each year on the project.

Jessica Millar, BS, is a second year graduate student in the lab. She generated the preliminary data showing the contribution of horizontal gene transfer to *Coxiella*'s biology. She already has two publications from the lab (one as first author), and another paper where she is the first author is under review currently. She will concentrate on understanding how horizontally derived genes drive *Coxiella*'s metabolism (*Specific Aim 2*). This project will be part of her thesis. The requested fund will facilitate a timely completion of her graduate degree.

Jonathan Gerhart, BS, is a first year graduate student in the lab. He is proficient at both bioinformatics and genomics. He recently assembled the genomes of a *Francisella* species present in ticks (he is the first author on the paper, which is currently under review). He will concentrate on assembling the genome of the *Coxiella* present in *Ornithodoros rostratus* (*Specific Aim 1*). This project will be part of his thesis. The requested fund will facilitate a timely completion of his graduate degree.

Undergraduate students

Melissa Holler and Todd Hinsch are two undergraduate researchers in the lab. They will each work 3 summer months each year on the project at $12/hr for approximately 69.5 hours per month. They will assist with tissue culture, *Coxiella* culture and molecular techniques such as PCR, RT-qPCR etc. They will also be trained on bioinformatics tools required to analyze high-throughput genomic and transcriptomic data. The summer support requested will allow the students to be fully immersed in the lab experience and not look for other jobs. New students will be hired to replace Melissa and Todd when they graduate.

Fringe Benefits

Portland State University's fringe benefit rates are budgeted per budgeting practices as follows:
- 30% of PI salary
- 30% of post-doctoral researcher salary
- 9% of GRAs' summer salary
- 9% of undergraduate students' summer salary

SERVICES & SUPPLIES

Supplies ($[redacted] funds are requested annually for project specific supplies.

Genome sequencing library preparation and Illumina HiSeq or NextSeq: $[redacted]
RNA-seq library preparation and Illumina NextSeq: $[redacted]
Molecular biology reagents (primers, SYBR green, probes etc.): $[redacted]
Enzymes and kits (Taq, T7 RNA polymerase, extraction kits etc.): $[redacted]
Tissue culture and *Coxiella* culture (plastics, media, fetal calf serum, antibiotics etc.): $[redacted]

Travel ($[redacted] $[redacted] is requested annually to support project personnel travel. These funds will allow PI or students to attend science meetings related to the proposed research each year, for example, the General Meeting of the American Society of Microbiology (ASM) or the Annual Meeting of American Society of Rickettsiology (ASR). Yearly meetings for students are a vital part of their training. Students will be required to present posters (or short talks when possible) at meetings. Both undergraduates and graduate students will be encouraged to attend.

Publication Costs ($[redacted] $[redacted] is requested annually to cover page charges for publication of results that are generated by this proposal. We will publish the results from this project in high impact journals such as *Genome Research*, *Genome Biology Evolution*, *PLoS Pathogens* and *mBio*.

Indirect Costs:
Indirect costs (F&A) at PSU are calculated based on Modified Total Direct Costs (first $[redacted] of the subcontract, minus tuition, equipment over $[redacted] at 48.5% rate over the project period per Portland State University's federally negotiated indirect cost rate agreement.

RESEARCH & RELATED BUDGET - Cumulative Budget

	Totals ($)	
Section A, Senior/Key Person		[redacted]
Section B, Other Personnel		[redacted]
Total Number Other Personnel	5	
Total Salary, Wages and Fringe Benefits (A+B)		[redacted]
Section C, Equipment		0.00
Section D, Travel		[redacted]
1. Domestic	[redacted]	
2. Foreign	0.00	
Section E, Participant/Trainee Support Costs		0.00
1. Tuition/Fees/Health Insurance	0.00	
2. Stipends	0.00	
3. Travel	0.00	
4. Subsistence	0.00	
5. Other	0.00	
6. Number of Participants/Trainees	0	
Section F, Other Direct Costs		[redacted]
1. Materials and Supplies	[redacted]	
2. Publication Costs	[redacted]	
3. Consultant Services	0.00	
4. ADP/Computer Services	0.00	
5. Subawards/Consortium/Contractual Costs	0.00	
6. Equipment or Facility Rental/User Fees	0.00	
7. Alterations and Renovations	0.00	
8. Other 1	0.00	
9. Other 2	0.00	
10. Other 3	0.00	
Section G, Direct Costs (A thru F)		[redacted]
Section H, Indirect Costs		[redacted]
Section I, Total Direct and Indirect Costs (G + H)		[redacted]
Section J, Fee		0.00

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PHS 398 Cover Page Supplement

OMB Number: 0925-0001

Expiration Date: 10/31/2018

1. Human Subjects Section

Clinical Trial? ❍ Yes ● No

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

2. Vertebrate Animals Section

Are vertebrate animals euthanized? ❍ Yes ● No

If "Yes" to euthanasia

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

❍ Yes ❍ No

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

3. *Program Income Section

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

❍ Yes ● No

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

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

Tracking Number: GRANT12198450

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4. Human Embryonic Stem Cells Section

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

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://grants.nih.gov/stem_cells/registry/current.htm. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

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

Cell Line(s) (Example: 0004):

5. Inventions and Patents Section (RENEWAL)

*Inventions and Patents: ❍ Yes ❍ No

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

*Previously Reported: ❍ Yes ❍ No

6. Change of Investigator / Change of Institution Section

❑ Change of Project Director / Principal Investigator

Name of former Project Director / Principal Investigator

Prefix:

*First Name:

Middle Name:

*Last Name:

Suffix:

❑ Change of Grantee Institution

*Name of former institution:

Tracking Number: GRANT12198450

Funding Opportunity Number: PA-16-200 . Received Date: 2016-06-20T20:28:55.000-04:00

PHS 398 Research Plan

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

Introduction	
1. Introduction to Application (Resubmission and Revision)	Introduction-R15-2016-resub.pdf
Research Plan Section	
2. Specific Aims	SpecificAims-R15-2016-resub.pdf
3. Research Strategy*	ResearchStrategy-R15-2016-resub-v10.pdf
4. Progress Report Publication List	
Human Subjects Section	
5. Protection of Human Subjects	
6. Data Safety Monitoring Plan	
7. Inclusion of Women and Minorities	
8. Inclusion of Children	
Other Research Plan Section	
9. Vertebrate Animals	
10. Select Agent Research	Select_agents-R15-2016-resub.pdf
11. Multiple PD/PI Leadership Plan	
12. Consortium/Contractual Arrangements	
13. Letters of Support	LOS_combined.pdf
14. Resource Sharing Plan(s)	Resource_sharing-R15-2016-resub.pdf
15. Authentication of Key Biological and/or Chemical Resources	Authentication_key_resources.pdf
Appendix	
16. Appendix	

Tracking Number: GRANT12198450
Funding Opportunity Number: PA-16-200. Received Date: 2016-06-20T20:28:55.000-04:00

INTRODUCTION TO APPLICATION

I am grateful to the reviewers for their critiques. I believe the revised proposal has been improved by addressing all their concerns (marked in blue).

Reviewer	Sig.	Invest.	Innov.	App.	Env.
One	3	1	4	2	1
Two	3	1	2	4	1
Three	2	1	2	2	1
Impact score: 28					

REVIEWER 1

Significance

Small number of annual cases reduces my enthusiasm. It is important to study this select agent because: (1) actual rates of *C. burnetii* infection are not known due to widespread underreporting, (2) *C. burnetii* causes epidemics such as the recent outbreak in the Netherlands that involved over 4,000 cases, (3) chronic Q fever has poor prognosis, and (4) current antimicrobial therapy is protracted (years) and generally ineffective.

The broader implications of this research are unclear. Our approach could be applied to other bacterial pathogens where human-specialized strains could be compared to avirulent symbionts. E.g., metabolic and virulence mechanisms in *Francisella* and *Rickettsia* could be compared to that of closely-related tick symbionts.

The impact of this application on the institution is not clear. This critique runs counter to compliments made by reviewer 2. We have addressed this under "*Impact of an AREA award on student research*" in the Facilities and Resources section. Additionally, a student-training plan is detailed at the end of each Aim.

Innovation

While examining horizontal gene transfer (HGT) is new in *Coxiella* research, it is a well-understood phenomenon. The methods seem appropriately chosen, but standard practice in the field. While HGT is well studied, our project is based on a novel concept that *C. burnetii* evolved from a tick-associated ancestor through massive HGT (>20% of genes). Additionally, our innovative approach will overcome the current difficulty in performing genome-scale functional studies in *C. burnetii*, and will define a novel tRNA-based virulence mechanism.

Approach

Use of KEGG and other databases to confirm the function of genes is overly optimistic. We don't expect this to be a problem because we are *not* utilizing the databases for identifying specific functions of each gene. Rather, we are interested in identifying *metabolic pathways* that contain pathogen-specific genes. Thus, a lack of functional information or misidentification of a few genes would not adversely impact this project.

Presence of $tRNA^{Glu}1$ will confound the ability to draw clear conclusions about the essentiality of heme biosynthesis. We don't expect $tRNA^{Glu}1$ to impact this project because as shown in **Figure 13** (Aim 3), the $tRNA^{Glu}2$-deletion strain has a clear growth defect. We will test whether the reduced growth is due to low heme production, and whether it is rectified by complementation, thus revealing the importance of heme biosynthesis.

REVIEWER 2

Significance and Innovation

No clear plan for prioritizing genes identified in Aims 1 and 2. We have added a prioritization plan in the revised application (please see **Table 1** under Innovation). We will prioritize: (1) pathways for the synthesis of critical metabolites that *C. burnetii* cannot obtain from host (e.g. heme, biotin), (2) pathways for metabolizing critical nutrients such as glutamate, and (3) proteins required for homeostasis (e.g. Na+/H+ antiporters).

Approach

Will the genes identified in Aim 1 be characterized in Aim 2? We will prioritize pathways that are common to both Aim 1 and Aim 2 i.e. pathogen-specific pathways that contain HGT-derived genes (please see **Table 1**).

How will you differentiate between genes required for metabolism from those required for entry, replication or general survival? We expect to identify pathways found only in the human pathogen, which contain HGT-derived genes. Of these, we will prioritize metabolic pathways (based on KEGG, MetaCyc etc.), but proteins required for entry, replication etc. will also be analyzed if time and budget permits (please see **Table 1**).

How will you show that the observed defects specifically manifest during intracellular growth. To identify infection-specific genes, we will compare the genes expressed in *C. burnetii* grown intracellularly to those grown in ACCM-2 medium. Additionally, transposon mutants will be tested in both ACCM-2 and in host cells to identify genes that are critical to intracellular growth.

What will be done with hypothetical genes? We expect these genes to be of low priority at this stage of the project. This should not be a problem because we have several high-quality targets that we expect to pursue.

SPECIFIC AIMS

The inhospitable environment within a lysosome-derived vacuole is the preferred growth medium for *Coxiella burnetii*, the etiological agent of human Q fever. The unique ability of *C. burnetii* to thrive in this acidic vacuole that contains noxious cationic peptides, reactive oxygen species, and lysosomal hydrolases is the key to its virulence. However, metabolic pathways critical to the pathogen's intracellular growth are *unknown*, mainly due to the unavailability of appropriate genome-scale approaches. This gap in knowledge is an important problem because it has *hindered* both the understanding of *C. burnetii*'s basic biology and pathogenesis, and the development of better therapies. Long-term combinatorial treatment with doxycycline and hydroxychloroquine (≥1.5 years) is the best available option; however, strains resistant to this treatment are prevalent. This select agent is found worldwide and can occasionally cause epidemics; e.g., a recent outbreak in the Netherlands involved ~4,000 human cases and caused the culling of over 50,000 goats (a reservoir).

Our *long-term goal* is to understand the molecular details of *Coxiella*'s distinctive physiology, and to apply this knowledge to developing novel therapeutic strategies. Towards attaining this goal, the *overall objective* of this application is to identify metabolic pathways that are vital to *C. burnetii*'s intracellular growth. Our *central hypothesis* is that *C. burnetii* evolved from a tick-associated ancestor by acquiring critical metabolic genes through horizontal gene transfer (HGT). Our hypothesis was formulated based on our previous work (Smith et al. 2015), which showed that the closest relatives of *C. burnetii* are avirulent bacteria with reduced metabolic capabilities present in ticks. The work by Duron et al. (2015) also supports this hypothesis. Based on these insights, we will use a *novel evolutionary genomics approach that will overcome the current technical difficulties* to identify metabolic processes critical to *C. burnetii*. The proposed research will be *impactful* because once metabolic processes important to *C. burnetii*'s intracellular growth are identified, new pharmacological agents that block these pathways can be developed to treat chronic Q fever more effectively.

To test our central hypothesis, in Specific Aims 1 and 2 we will use two complementary genome-scale approaches to define critical metabolic processes in *C. burnetii*, and in Specific Aim 3, as a proof of principle, we will experimentally validate the importance of heme biosynthesis to *Coxiella*'s physiology.

1. Identify metabolic pathways that distinguish *C. burnetii* from tick-associated *Coxiella*. Because *Coxiella* species present in ticks do not replicate within a lysosome-derived vacuole, our *working hypothesis* is that genes critical to *C. burnetii*'s unique intracellular physiology will not be present in tick-associated *Coxiella*. We will sequence the complete genome of *C. burnetii*'s closest known relative—a *Coxiella* species present in the soft tick *Ornithodoros rostratus*, and by comparative genome analyses, we will identify metabolic genes and pathways unique to *C. burnetii*.

2. Define metabolic pathways that are critical to *C. burnetii*'s intracellular growth. Superfluous genes are lost from obligate intracellular bacteria such as *C. burnetii*. This is especially true for genes acquired via HGT, which will be retained only if they improve *C. burnetii*'s fitness. Consequently, we *hypothesize* that horizontally derived genes are critical to *C. burnetii*'s physiology. We will utilize phylogenetic approaches to identify HGT-derived metabolic genes among those identified in Aim 1. Using RNA-seq and transposon-insertion libraries, we will validate their importance to *C. burnetii*'s intracellular growth.

3. Determine the importance of heme biosynthesis to *C. burnetii's* intracellular growth. *C. burnetii*'s heme biosynthesis pathway contains HGT-derived genes, and this pathway is not present in *Coxiella* symbionts, and we established that *C. burnetii* could not use external heme. We therefore *hypothesize* that heme biosynthesis is crucial to *C. burnetii*'s intracellular growth and can serve as an example for the efficacy of our novel approach. We will confirm the importance of heme biosynthesis to *Coxiella* by assaying heme production and intracellular growth in wild-type and heme pathway-deficient strains.

At the successful completion of this project, *expected outcomes* include elucidation of the evolutionary history of this enigmatic pathogen, and identification of metabolic pathways that are important to *Coxiella*'s intracellular growth and virulence. Results are expected to have a *positive impact* because it will provide a *useful model for identifying key metabolic* processes in other human pathogens with closely related symbionts such as *Rickettsia* and *Francisella*. Additionally, non-redundant proteins in critical metabolic pathways, e.g. HemA and HemL in heme biosynthesis, are ideal *candidates for developing new anti-Coxiella agents.*

RESEARCH STRATEGY

(A) SIGNIFICANCE

Importance of the problem: *Coxiella burnetii* is the etiological agent of a flu-like illness (acute Q fever) and a chronic disease commonly manifested as endocarditis (Maurin and Raoult 1999). CDC has classified *Coxiella* as a select agent due to its past use as a bioweapon, environmental stability, aerosol transmission and extremely low infectious dose [ID50 of one to ten bacteria in a guinea pig model (Tigertt et al. 1961)]. Human Q fever is a nationally notifiable disease in the U.S. since 1999, however the true rate of infection is not known due to widespread underreporting (CDC 2002). Untreated chronic Q fever is associated with high mortality rate (>60%) (Karakousis et al. 2006), and *Coxiella* is one of the commonest causes of culture-negative infective endocarditis (Million et al. 2010). *C. burnetii* is found worldwide, and can cause epidemics, such as the recent one in the Netherlands where thousands of people were infected (van der Hoek et al. 2012). It has also been detected among U.S. military personnel (Faix et al. 2008), and its high prevalence in US environmental samples (Kersh et al. 2010; Loftis et al. 2010) highlight the *urgent need to understand its biology and virulence mechanisms*. Acute infections are treatable but even with the recommended 3-week doxycycline treatment, acute Q fever can transform into chronic endocarditis (Fenollar et al. 2006). Chronic *Coxiella* infections are very difficult to treat because it requires prolonged antibiotic regimen: a combination of doxycycline and hydroxychloroquine (1.5 to 3 years), or doxycycline and fluoroquinolone (3 to 4 years) (Gould et al. 2012). However, strains resistant to doxycycline are prevalent (Rouli et al. 2012). *A critical barrier to understanding Coxiella's basic biology and molecular pathogenesis, and to developing more effective therapies is the lack of knowledge about metabolic processes that are vital to Coxiella's growth within human cells.*

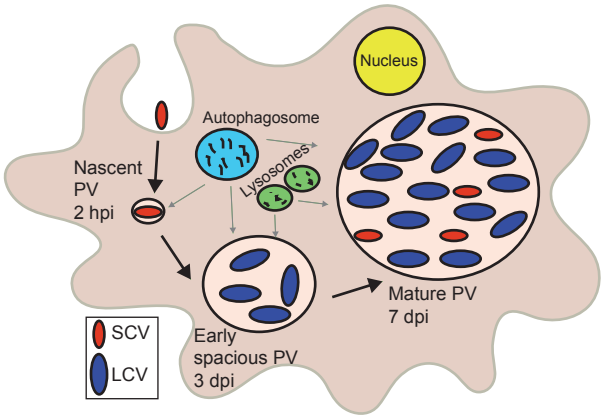


Figure 1. Biogenesis of *C. burnetii* parasitophorous vacuole (PV).

How the proposed project will advance one or more broad fields: *C. burnetii* is an intracellular pathogen that has a biphasic life cycle (**Figure 1**). The bacterium survives in the environment as a metabolically quiescent small cell variant (SCV). *C. burnetii* has occasionally been isolated from ticks, but most human infections occur through inhalation of aerosols contaminated with SCV originating from farm animals. Shortly after entering into a host cell (typically an alveolar macrophage in human infections), SCV transforms into a metabolically active large cell variant (LCV) within a lysosome-derived parasitophorous vacuole (PV) (Voth and Heinzen 2007). Over the next 5-6 days LCVs divide within the acidic vacuole (pH ~4.5) to large numbers and eventually transform into SCVs, which are released upon lysis of the host cell (Minnick and Raghavan 2012). Its closest relatives are avirulent bacteria found associated with ticks (Smith et al. 2015). These *Coxiella* species do not have the ability to replicate within human cells (Duron et. 2015). It is not understood how a tick-associated non-pathogenic ancestor evolved into a human pathogen, and the metabolic capabilities that allow *C. burnetii* to thrive in the harsh conditions within the PV are also not understood (**Figure 2**). We postulate that elucidating the evolutionary process that resulted in the emergence of *C. burnetii* is the key to unlocking its distinctive physiology and virulence strategy. The proposed research is *significant* because in addition to *(1)* uncovering the evolutionary history of *C. burnetii,* we will for the first time, *(2)* identify metabolic processes that are critical to the pathogen's intracellular growth, thereby advancing the field considerably by *(3)* improving our knowledge about its basic physiology and *(4)* providing potential therapeutic targets. Importantly, the proposed project provides *(5)* a model approach for identifying genes involved in host adaptation, which could be applied to other pathogens such as *Francisella tularensis* or *Rickettsia* spp. where avirulent symbionts could be compared to virulent human-specialized strains. Furthermore, *(6)* we will examine the importance of heme, which is synthesized from a $tRNA^{Glu}$ that was acquired horizontally by *Coxiella*. Because other pathogenic bacteria such as *Corynebacterium* and *Clostridium* encode multiple $tRNA^{Glu}$ isoacceptors, this novel virulence strategy could be widespread among human pathogens.

Figure 2. Stages in *C. burnetii* infection (modified from Garber 1960). Gap in knowledge is depicted in red.

(B) INNOVATION

How the proposed research seeks to shift current research paradigms: Bacterial infection involves three broad stages (**Figure 2**, previous page). Current research into *Coxiella*'s pathogenicity is mainly focused on entry (Stage 1), and on manipulation of the host cell environment (Stage 3) (e.g., Weber et al. 2013; Newton et al. 2014; Larson et al. 2015, Graham et al. 2015). This bias has left a *large void in our understanding of Coxiella's intracellular metabolism* (Stage 2), which has been fine-tuned to replicate in the harsh environment within a lysosome-derived vacuole. As antibiotic resistance continue to rise among pathogens, it is critical that researchers explore unconventional antimicrobial targets. An under-explored opportunity is the simple premise that a bacterium needs to be metabolically active during infection (Brown et al. 2008). Louis Pasteur originally proposed this concept in his model of the body as a culture vessel (Pasteur 1878); however, very few studies have explored this concept to develop new pharmacological agents that inhibit bacterial growth within hosts. Because *C. burnetii* replicates within an intracellular niche that is lethal to all other bacteria, we postulate that uncovering its metabolic capabilities is the key to understanding its virulence strategies and to developing new therapeutic agents. The proposed research will *shift the current paradigm* in *Coxiella* research because we expect to identify, for the first time, the metabolic pathways that are critical to *Coxiella*'s distinct intracellular physiology.

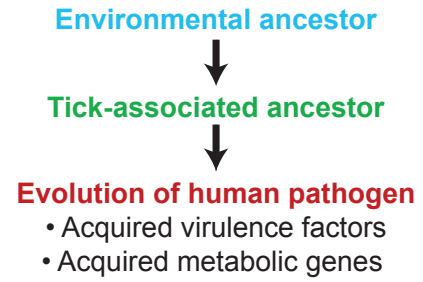
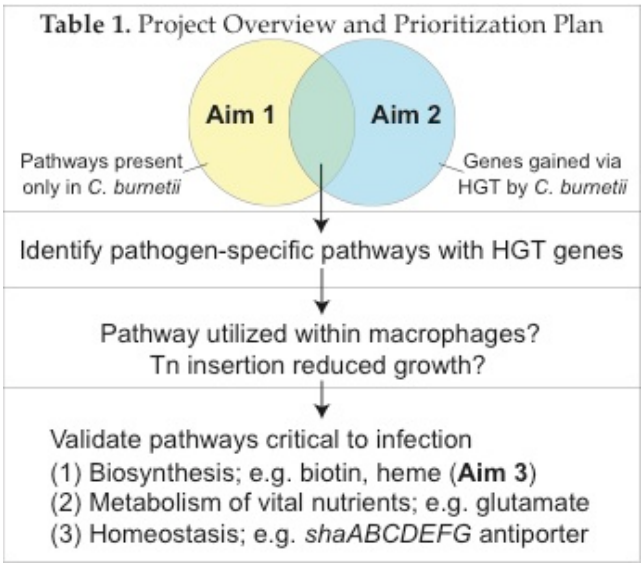


Figure 3. Emergence of *C. burnetii*.

Novel theoretical concepts, innovative approaches to be developed or used: Currently, no efficient genome-scale methods are available to identify metabolic genes that are important to *Coxiella*'s intracellular growth. A targeted gene deletion system is available (Beare et al. 2012), but this method is labor intensive and is not suitable to be applied at a genome-wide scale. Random transposon (Tn) insertion mutants of *C. burnetii* have been used to identify genes important for host cell invasion (Martinez et al. 2014, Newton et al. 2014). However, because Tn insertions in essential metabolic genes are likely lethal, it is hard to determine whether a gene has not been targeted by Tn because the gene is essential or due of a lack of saturation. Additionally, genetic systems to generate conditional mutants that circumvent the lethality of mutations in essential genes are also not available. *To overcome these limitations we have devised an innovative evolutionary-genomics approach* that will identify most metabolic pathways that are vital to *C. burnetii*'s intracellular growth. Our approach is based on preliminary data that indicate that *C. burnetii* evolved from a tick-associated ancestor by acquiring genes through horizontal gene transfer (HGT) (**Figure 3**). *This is a novel concept in our understanding of bacterial pathogenesis because no other pathogen is known to have evolved in this manner*. We will compare the genomes of *C. burnetii* to that of a closely related avirulent tick-associated *Coxiella* to identify pathogen-specific metabolic genes. In parallel, we will identify metabolic genes that were acquired via horizontal gene transfer (HGT) (**Table 1**). By comparing these two sets of genes we will generate a list of metabolic pathways that are instrumental in *C. burnetii*'s ability to grow intracellularly, and are hence ideal candidates for disruption using new therapeutic strategies. As a first step, we will examine the importance of heme biosynthesis to *Coxiella*'s intracellular growth because this pathway is unique to the human pathogen and contains horizontally derived genes, including a $tRNA^{Glu}$. The use of an alternate tRNA to promote infection represents a *novel pathogenic mechanism*. Collectively, this project is *innovative* because it *(1)* utilizes a novel approach that overcomes the current limitations in studying *Coxiella* genes at a genome-wide scale, *(2)* is expected to identify metabolic pathways that are important for *Coxiella*'s intracellular growth, *(3)* is based on a novel concept that the virulent strain evolved via HGT from an avirulent tick-associated ancestor, *(4)* is expected to make new targets available for developing novel therapeutic agents, and *(5)* will reveal a novel tRNA-acquisition-based virulence mechanism that could be widespread among other pathogens. A project overview and our prioritization plan are shown in **Table 1**.

Table 1. Project Overview and Prioritization Plan



(C) APPROACH

Specific Aim 1. Identify metabolic pathways that distinguish *C. burnetii* from tick-associated *Coxiella*.

Background and Justification

Within macrophages, engulfed pathogens are transported inside phagosomes that later fuse with lysosomes to generate the phagolysosome — a very harsh environment due to low pH, high concentration of lysosomal hydrolases and presence of cationic peptides (Kinchen and Ravichandran, 2008; Flannagan et al. 2009). Pathogenic bacteria have evolved strategies to circumvent the mature lysosome. For instance, *Legionella pneumophila* blocks maturation of phagosomes into phagolysosomes, and *Listeria monocytogenes* escape into the cytoplasm from phagosomes before lysosomal fusion (Flannagan et al., 2009). Unlike other pathogenic bacteria, *C. burnetii* has evolved the ability to thrive in a parasitophorous vacuole (PV) that is derived from the fusion of phagosomes with lysosomes (Voth and Heinzen, 2007; Alix et al., 2011) (**Figure 1**). The only other human pathogen that has evolved this capability is the eukaryotic parasite *Leishmania* (Real et al. 2010). We recently showed that *C. burnetii* and *L. mexicana* use disparate strategies for growth within their respective PVs (Millar et al. 2015). *The metabolic pathways that drive C. burnetii's growth within PV are unknown*, even though it is fundamental to the bacterium's physiology and pathogenicity.

The evolutionary origin of *C. burnetii* is not clearly understood. The closest relatives of *C. burnetii* are found in ticks (Smith et al. 2015; Duron et al. 2015) (**Figure 4**). *Coxiella* spp. found in ticks are avirulent, cannot infect mammalian cells, and are unable to grow in a culture medium that supports robust growth of *C. burnetii* (Smith et al. 2015; Gottlieb et al. 2015; Duron et al. 2015). These observations suggest that despite their close evolutionary relationship, the human pathogen and the tick-associated strains have different metabolic capabilities. Our *working hypothesis* is that genes critical to *C. burnetii*'s unique lifestyle will not be present in closely related tick-associated coxiellae. To test our hypothesis and thereby to accomplish the objective of this Aim, we will sequence the genome of a *Coxiella* present in the soft-bodied tick *Ornithodoros rostratus*, which is the closest known relative of *C. burnetii* (Almeida et al. 2012) (**Figure 4**). We will use a comparative-genomics approach to identify metabolic pathways that are exclusive to *C. burnetii*. By successfully completing this aim, we expect to reveal the physiological capabilities that facilitated the emergence of a human pathogen from a tick-associated ancestor.

Preliminary Studies and Feasibility

We are well versed at studying bacterial genomes and transcriptomes (Raghavan et al. 2011a, Raghavan et al. 2011b, Raghavan et al. 2012, Raghavan et al. 2015; Warrier et al. 2014; Millar et al. 2015). We recently sequenced the whole genome of a *Coxiella*-like bacterium from the Lone Star tick *Amblyomma americanum* (Smith et al. 2015). This was the first genome of a tick-associated *Coxiella* to be sequenced. We will use a similar strategy to sequence the genome of the *Coxiella* species present in *O. rostratus*. To prepare for this project we procured 35 adult ticks from an *O. rostratus* lab colony maintained by Dr. Marcelo Labruna at the University of Sao Paulo, Brazil (see Letter of Support). Dr. Labruna's group previously showed that the *Coxiella* species present in this *O. rostratus* colony is the closest known relative of *C. burnetii* (Almeida et al. 2012). We have successfully isolated good quality genomic DNA from the ticks and are all set to perform the experiments described below.

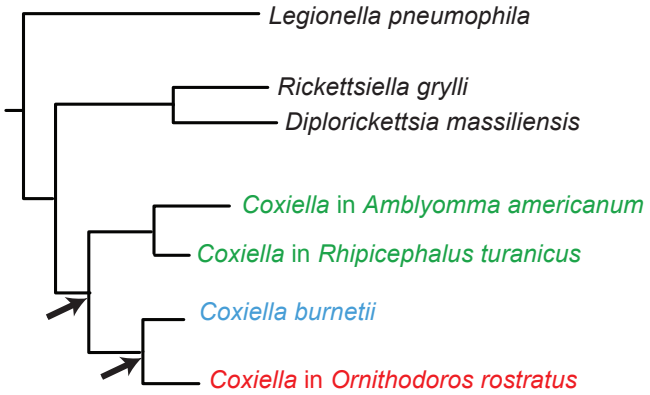


Figure 4. Two strains from hard ticks have sequenced genomes (green). We will sequence the strain from a soft tick (in red), which is more closely related to *C. burnetii*.

Research Design

(1) Sequence, assemble and annotate the genome of *Coxiella* present in *O. rostratus*.

We will submit genomic DNA (~1µg) extracted from one adult tick to OHSU Massively Parallel Sequencing Shared Resource for high-throughput sequencing (see Letter of Support). We will sequence each DNA sample on one lane of Illumina NextSeq (300 cycle, paired-end). We will assemble the genome using Velvet and IDBA genome assembly programs (Zerbino and Birney 2008; Peng et al. 2012), and close the

gaps using PCR and Sanger sequencing, as we described in Smith et al. (2015). We will annotate the genome using the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al. 2013), and manually check these annotations to produce the final annotated genome. We will deposit the genome sequence in GenBank, and the sequencing reads will be made freely available to the scientific community.

(2) Perform comparative genomics to identify pathogen-specific metabolic genes. Currently, seven completely sequenced genomes of *C. burnetii* strains are available. We will align the new genome to all seven *C. burnetii* genomes using Mauve aligner (Darling et al. 2010) to identify pathogen-

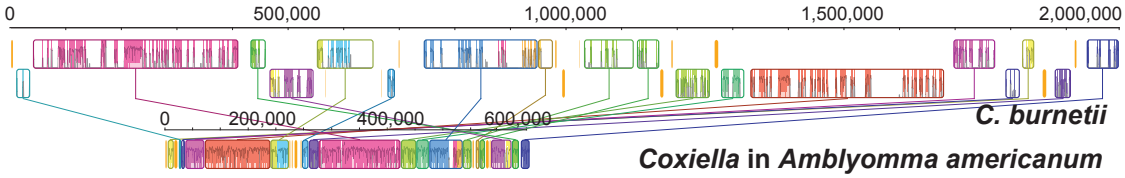


Figure 5. Alignment of *Coxiella* genomes. Regions in white in *C. burnetii* genome (on top) denote genes present only in the human pathogen. Modified from Smith et al. 2015.

specific genes (**Figure 5**). We will also use reciprocal BLAST, as described previously (Raghavan et al. 2012; Raghavan et al. 2015; Smith et al. 2015) to identify pathogen-specific genes i.e. *C. burnetii* genes without orthologs in tick associated *Coxiella*. By comparing the *C. burnetii* genomes, we will identify pathogen specific metabolic genes that are conserved in all strains of *C. burnetii*.

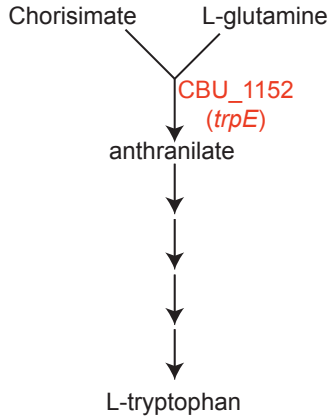
To identify the metabolic pathways to which each gene belongs, we will perform a Gene Ontology (GO) analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al. 2009). This analysis will bin each protein into functional groups based on the GO terms associated with each one. In parallel, we will use the Kyoto Encyclopedia of Genes and Genomes (KEGG), and the MetaCyc database to corroborate their metabolic functions (Kanehisa et al. 2015; Caspi et al. 2012). We have previously utilized these tools (Raghavan et al. 2015; Smith et al. 2015; Millar et al. 2015). For instance, in a previous study (Smith et al. 2015), we identified the gene CBU_1152, present in *C. burnetii* but not in *A. americanum*-associated *Coxiella,* as belonging to tryptophan biosynthesis pathway (**Table 2**).

Table 2. Identification of metabolic functions of pathogen-specific genes

Gene	GO term	GO description
CBU_1152	GO:0000162	Tryptophan biosynthetic process
	GO:0006568	Tryptophan metabolic process

KEGG Description
KEGG Orthology (KO) [BR:cbu00001]
Metabolism
Overview
01230 Biosynthesis of amino acids
Amino acid metabolism
00400 Phenylalanine, tyrosine and tryptophan biosynthesis
Enzymes [BR:cbu01000]
4. Lyases
4.1 Carbon-carbon lyases
4.1.3 Oxo-acid-lyases
4.1.3.27 anthranilate synthase

MetaCyc Pathway



Expected Outcomes

(1) We expect to sequence and annotate the complete genome of a *Coxiella* spp. present in *O. rostratus*. The availability of the first *Coxiella* genome from a soft tick, along with the two genomes from hard ticks (Smith et al. 2015; Gottlieb et al. 2015) will be a great resource to understand the evolution of this enigmatic pathogen. By studying these three genomes and by comparing them to *C. burnetii* genomes, *we expect to uncover the step-wise evolution* of the human pathogen by acquisition of genes through HGT (**Figure 3** and marked by arrows in **Figure 4**).

(2) We expect to define a set of *genes that are present only in the pathogenic strains* of *Coxiella*, and to identify the metabolic processes that contain these pathogen-specific genes. We will also identify the differences in metabolic capabilities of various *C. burnetii* strains, thereby gaining new insights into strain-specific differences in pathogenicity.

(3) Student training. Todd Smith, a previous undergraduate student sequenced the genome of the *Amblyomma*-associate *Coxiella* (Smith et al. 2015). Jonathan Gerhart, a first year graduate student will perform the genome assembly and annotation described in this Aim. I will work with him to achieve the objectives of this Aim in a timely manner. In addition, an undergraduate student Melissa Holler will work with us

to learn programming (e.g. PERL, PYTHON) and the use of bioinformatics tools described above. Melissa plans to pursue a Masters degree in Bioinformatics at the joint program offered by PSU and OHSU.

Potential Problems and Alternative Strategies

We are highly experienced at sequencing genomes and performing comparative genomics to identify species-specific genes (Raghavan et al. 2012; Raghavan et al. 2015; Smith et al 2015; Millar et al. 2015). We don't expect any major problems with this aim; however, couple of minor roadblocks might arise for which we have alternate strategies in place:

(1) We expect our genomic DNA samples to be composed mostly of tick DNA. We don't expect this to be a problem because we were able to assemble the genome of the *Coxiella* in *A. americanum* using only ~5% of sequencing reads (Smith et al. 2015). However, if we don't obtain enough reads to assemble the *Coxiella* genome from our first attempt, we will resequence the sample in another lane of Illumina NextSeq, which should provide us with enough reads to allow efficient assembly of the new *Coxiella* genome.

(2) Because bacteria contain repeat sequences that preclude the assembly of genome sequences into a single circular chromosome, we expect the initial genome assembly to be composed of multiple fragments (contigs). For instance, in our previous study (Smith et al. 2015), we initially had six contigs that were stitched together using PCR and Sanger sequencing. If we are unable close the new genome using this approach, we will resequence the DNA using either Illumina Mate-Pair or PacBio technologies that produce long reads that bridge repeating sequences, thereby allowing us to close the genome.

(3) Using GO, DAVID, KEGG, and MetaCyc databases, we expect to identify the metabolic pathways that contain most of the pathogen-specific genes. However, it is possible that we will not be able to place all genes into specific pathways (e.g., hypothetical genes), and it is possible that some of the genes might be performing functions that are different from those recorded in the databases. However, we don't expect this to be a major hurdle because our goal is to identify metabolic pathways — not specific genes — that are unique to the human pathogen. Thus, a lack of functional information or misidentification of a few genes would not adversely impact this project.

Specific Aim 2. Define metabolic pathways that are critical to *C. burnetii*'s intracellular growth.

Background and Justification

When the genomes of *C. burnetii* were sequenced, it became clear that several genes were acquired via HGT, including a Type Four Secretion System (TFSS) — a major virulence factor (Seshadri et al. 2003; Beare et al. 2009). Interestingly, in addition to TFSS, *C. burnetii* encodes several genes for a type IV pilus. A related type IV pilus enables *Acinetobacter baumannii* to acquire DNA from the environment with high efficiency (Smith et al. 2007). It is unlikely that the type IV pilus has the same function in *C. burnetii* because some of the *pil* genes in *C. burnetii* have become pseudogenes (Beare et al. 2009). However, it is plausible that at an earlier stage of *C. burnetii*'s evolution, a functional type IV pilus endowed it with the ability to acquire foreign genes with high efficiency, thereby enabling its shift from a tick-associated bacterium to a zoonotic human pathogen. *Although foreign-origin genes are important to C. burnetii's emergence and virulence, no systematic study has been undertaken to understand the origins of all C. burnetii genes.*

Unlike eukaryotic genomes, which contain large fractions of nonfunctional DNA (e.g., >80% of human genome), bacterial genomes are tightly packed with functional genes due to elimination of nonfunctional sequences (Moran 2002). In bacteria there is a bias towards DNA deletion over insertion (Mira et al. 2001); thus, DNA is retained in a bacterial genome only if selection is acting effectively to preserve it. Many biosynthetic genes and pathways are rendered redundant in host-associated bacteria such as *C. burnetii* because they could obtain the corresponding metabolic intermediates from hosts (Ochman and Moran 2001). These superfluous genes will be deleted over time due to lack of selection acting to maintain them, thereby resulting in a small genome; e.g., *C. burnetii*'s genome is only ~2 million bp compared to *E. coli*'s, which is ~5 million bp. Consequently, genes acquired through HGT will only be retained in *C. burnetii*'s genome if they improve its fitness (Lawrence 1999). Based on this information, we <u>*hypothesize*</u> that genes acquired via HGT are being retained in *C. burnetii*'s reduced genome because they play important roles in *Coxiella*'s biology. We will perform a thorough evolutionary analysis of all protein-coding genes in *C. burnetii* to determine the ones that are of foreign origin. The HGT-derived genes could be beneficial during *C. burnetii*'s growth in it primary reservoir (farm animals), or during its environmental phase, or during its growth with-

in human cells. To identify HGT genes that are functional during *C. burnetii*'s intracellular growth, we will analyze gene expression within human macrophages using RNA-seq. We will also screen Tn-insertion libraries to find strains with Tn-insertions in critical genes.

Preliminary Studies and Feasibility

(1) Characterization of horizontally acquired parasitic elements in *C. burnetii*. We have a great deal of experience in investigating the evolutionary histories of genes in *C. burnetii*. We previously published several papers that detail the origin and possible functions of group I introns, a homing endonuclease, and an intein present in *Coxiella* (Raghavan et al. 2007, Raghavan et al. 2008, Raghavan et al. 2009; Raghavan and Minnick 2009; Minnick et al. 2010; Hicks et al. 2011).

(2) Detection of genes that were potentially acquired from distant phyla in *C. burnetii*. To identify the closest orthologs of *C. burnetii* proteins, we performed a bidirectional BLAST analysis with the NCBI nr database (Wolf and Koonin 2012). To consider a protein to be orthologous, we applied a minimum cutoff of 70% coverage, and 30% amino acid identity, as described previously (Caro-Quintero et al. 2012). We detected 357 genes in *C. burnetii* that were putatively acquired from organisms outside of Gammaproteobacteria (*Coxiella* belongs to this *class*) (**Figure 6A**). This is a very conservative list because it

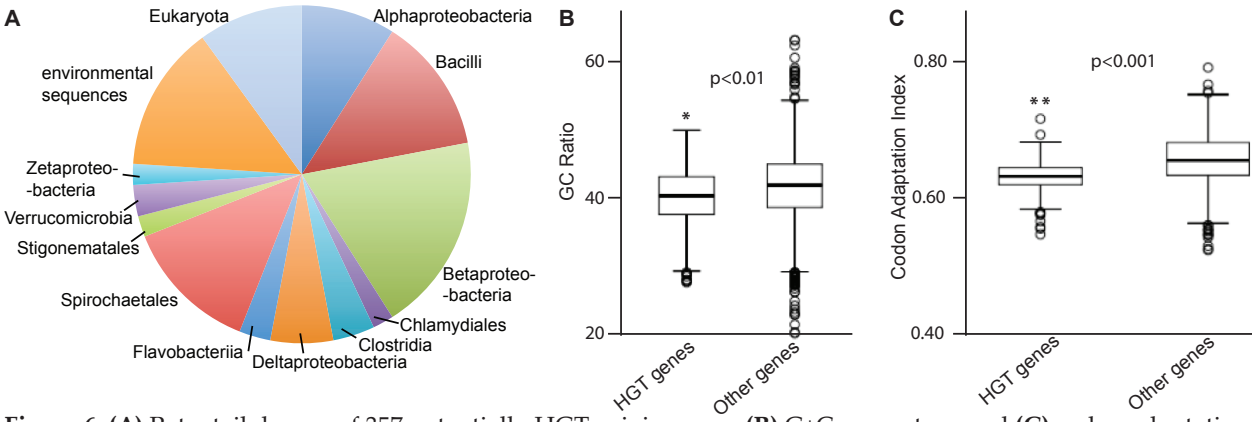


Figure 6. (A) Potentail donors of 357 potentially HGT-origin genes. **(B)** G+C percentage and **(C)** codon adaptation index for these genes are significantly diffrent from the rest of the genes.

does not include any known virulence factors such as the TFSS, or any gene that might have been gained from another Gammaproteobacteria. Incredibly, even with these omissions, ~20% of *C. burnetii*'s proteins (357/1823) appear to be horizontally derived. This is in contrast to most other bacterial genomes where <10% of the proteins are of foreign origin (Koonin et al. 2001). The G+C ratio and codon usage of horizontally acquired genes are typically different from the rest of the genome (Lawrence and Ochman 1997). Our analyses showed that in comparison to rest of the genes in *C. burnetii*, these two measures were significantly different for the 357 putative HGT genes (**Figures 6B and 6C**).

(3) Acquisition of a fatty acid biosynthesis operon from a Spirochete. While the BLAST best match approach is a useful first-pass discovery tool, phylogenetic analyses are required to confirm gene histories (Koski and Golding 2001). To this end, we analyzed a cluster of genes in *C. burnetii* that appear to have been acquired from a Spirochete. This putative operon contains fatty acid biosynthesis genes (*fabHAZB*) and their closest orthologs are present as a similar operon in *Spirochaeta africana* (**Figure 7A**, next page). An IS1111A transposase (CBU_0040) is seen next to these genes in *C. burnetii* suggestive of a role for a transposon in the horizontal acquisition of the genes. We confirmed its HGT origin by building a Bayesian tree for CBU_0038. As shown in **Figure 7B** (next page), the *C. burnetii* gene clustered with genes in *S. africana* and several Deltaproteobacteria, with the exclusion of genes from members of Legionellales (*Coxiella* belongs to this *order*). Furthermore, we confirmed that these genes are functional during intracellular growth by performing RNA-seq on *C. burnetii* growing within Vero (monkey kidney epithelium) cells (**Figure 7C**, next page).

Research Design

(1) Define the origins of protein-coding genes in *C. burnetii*. Both parametric and phylogenetic methods are commonly used to identify horizontally acquired genes in a genome. Parametric methods utilize inherent characteristics of a genome sequence such as G+C ratio, oligonucleotide frequency or codon usage to detect HGT, whereas phylogenetic methods identify HGT by detecting genes with evolutionary his-

tories that are inconsistent with the evolutionary history of the organism (Hooper and Berg 2002; Dufraigne et al. 2005; Vernikos and Parkhill 2006; Ravenhall et al. 2014). As shown in **Figure 6** and **Figure 7**, we have the expertise to use both methods to confirm the evolutionary histories of all genes in *Coxiella*. We will align orthologous sequences using MUSCLE (Edgar 2004), with regions of poor alignment masked using Gblocks (Talavera and Castresana 2007), and for phylogeny estimation, we will build Maximum-likelihood and Bayesian trees utilizing RAxML (Stamatakis et al. 2008) and PhyloBayes (Lartillot et al. 2013), respectively. For parametric analyses we will utilize in-house PERL and PYTHON scripts (Raghavan et al. 2012).

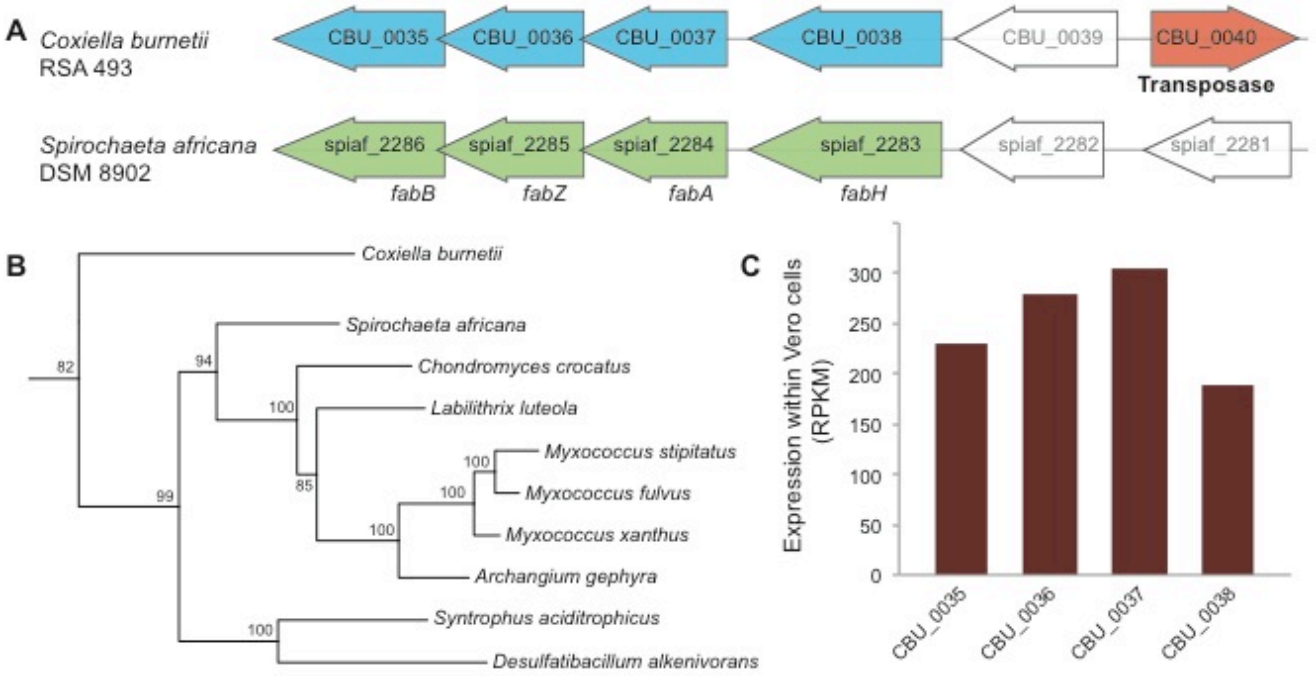


Figure 7. (**A**) The closest orthologs of *C. burnetii* fatty acid biosynthesis genes (blue) are present in *S. africana* (green) A transposase (red) flanks this region in *C. burnetii*, suggesting *en bloc* HGT of the genes. (**B**) A Bayesian tree showing the closest neighbors of CBU_0038. (**C**) HGT-derived genes are expressed while growing within Vero cells.

(2) Identify metabolic pathways that contain horizontally acquired genes. We will identify *C. burnetii*'s metabolic pathways that contain horizontally acquired genes using GO, DAVID, KEGG and MetaCyc as described in Aim 1 (**Table 2**). *By comparing pathogen-specific metabolic pathways identified in Aim 1 to those that contain HGT genes (Aim 2), we expect to determine the metabolic processes that are critical to C. burnetii's biology* (**Table 1**).

(3) Confirm the importance of metabolic pathways during intracellular growth using RNA-seq and transposon-insertion libraries. To validate the utility of metabolic pathways during intracellular growth, we will analyze the expression of *C. burnetii* genes within human macrophages using RNA-seq, as we have described before (Warrier et al. 2014; Millar et al. 2015). Briefly, we will infect human monocyte THP-1 cells (TIB-202; ATCC) differentiated into adherent, macrophage-like cells with *C. burnetii* RSA 493 NMII at a multiplicity of infection (MOI) of 25. We will collect total RNA at 3, 5, and 7-day post infection and prepare Illumina mRNA sequencing libraries from three independent infections as described previously (Warrier et al. 2014; Millar et al. 2015). As a control, we will use *C. burnetii* grown similarly in ACCM-2 for 3, 5, and 7 days. Libraries will be pooled and sequenced on a lane of Illumina NextSeq, and gene expression will be measured as described previously (Raghavan et al. 2011; Raghavan et al. 2012; Raghavan et al. 2015).

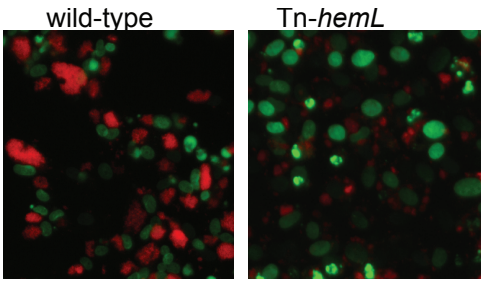


Figure 8. Tn insertion in *hemL* reduced intracellular growth significantly. Host nuclei shown in green and *C. burnetii* PVs in red. Data from Martinez et al. 2014.

Construction of loss-of-function deletion strains is the gold standard approach to validating the utility of a gene. However, in *C. burnetii*, targeted gene deletion is currently technically challenging and not expandable to the whole genome. An alternate approach is to use transposon-insertion mutants; however, because loss of function of essential meta-

bolic genes due to transposon insertion will most likely be lethal, we will utilize strains in which a transposon insertion has caused only a partial loss of gene function. For example, we procured a *C. burnetii* strain with a transposon inserted in the 3' end (1287/1320 bp) of the *hemL* gene from our collaborator Dr. Matteo Bonazzi (CNRS, France). The intracellular growth of this strain is significantly lower than that of the wild-type strain (**Figure 8**, previous page), indicating that HemL, and hence heme biosynthesis, is important to *C. burnetii*'s physiology (also see Aim 3). We will search transposon-insertion libraries available with Dr. Bonazzi and with Dr. James Samuel (Texas A&M University) to identify such Tn-insertion mutants to validate the utility of metabolic pathways to *C. burnetii*'s intracellular growth (see Letters of Support).

Expected Outcomes

(1) We expect to identify most horizontally derived genes in *C. burnetii*. This will be a big step in our understanding of the evolution of *C. burnetii* from a tick-associated ancestor. Our approach could be applied to investigate the evolution of other human pathogens that have closely related arthropod symbionts, e.g. *Rickettsia* spp., and *Francisella tularensis*.

(2) We expect to identify several metabolic pathways that contain horizontally acquired genes. Because these foreign-origin genes are being maintained in the reduced genome of *C. burnetii*, we expect them (e.g. heme and fatty acid biosynthesis pathways) to contribute towards organismal fitness, i.e., most of them will be important to *Coxiella*'s biology.

(3) By analyzing gene expression and Tn-insertion mutants, we expect to validate the importance of pathogen-specific, and HGT gene-containing pathways to *Coxiella*'s intracellular growth. Additionally, by comparing the expression of each gene in ACCM-2 vs. THP-1 cells, we will be able to identify genes that are induced inside the host cell. Understanding the metabolic pathways that are critical to *C. burnetii* while growing within human cells will be a major breakthrough in *Coxiella* research. We expect this new knowledge to lead to the unraveling of the pathogen's basic biology and virulence mechanisms, and to provide new targets for the development of novel therapeutic agents. We expect to identify several genes and pathways that are potentially important to *Coxiella*'s intracellular physiology and pathogenicity. *For validation, we will prioritize (1) pathways for the synthesis of critical metabolites that C. burnetii cannot obtain from host (e.g. heme, biotin), (2) pathways for metabolizing critical nutrients such as glutamate, and (3) proteins required for homeostasis (Na^+/H^+ antiporters)* (**Table 1**).

(4) Student training. Jessica Millar, a second year graduate student will accomplish the phylogenetic analyses described above. She generated most of the preliminary data that indicated the importance of HGT to *Coxiella*'s physiology. She will also perform the RNA-seq experiments as part of this Aim. She is well versed at using high-throughput sequencing to analyze gene expression. We have already published a paper that utilized RNA-seq extensively (Millar et al. 2015). Abraham Moses who joined my lab as an undergraduate and is now a Research Associate will perform the analyses on Tn-insertion mutants. He has worked on *Coxiella* for three years and plans to join my lab as a graduate student in couple of years.

Potential Problems and Alternative Strategies

We are confident about successfully achieving the goals of this aim because we have published several papers using the approaches proposed above (for e.g., Raghavan et al 2007; Raghavan et al. 2008; Raghavan et al. 2010; Raghavan et al. 2012; Smith et al. 2015; Millar et al. 2015). From that experience, we expect the following potential impediments for which we have alternate strategies in place:

(1) In our preliminary analysis, in addition to the 357 genes potentially acquired from non-Gammaproteobacteria, we detected 75 genes that were putatively obtained from members of Gammaproteobacteria. However, the phylogenetic neighborhood that contains *Coxiella* within Gammaproteobacteria is sparsely represented — *Legionella*, *Rickettsiella*, and *Diplorickettsia* are the only bacteria that are somewhat closely related to *Coxiella*. This lack of data makes it hard to identify genes acquired by *C. burnetii* from other Gammaproteobacteria. We will collaborate with Dr. Joseph Gillespie (University of Maryland) to try and resolve the evolutionary origins of such genes (see Letter of Support). Dr. Gillespie is an expert in identifying gene origins. He was involved in generating the most comprehensive phylogeny of Gammaproteobacteria (Williams et al. 2010), and assembled the database PATRIC (Gillespie et al. 2011). Even if we are not able to detect all genes that were acquired from Gammaproteobacteria, we still have enough genes (357) that were putatively gained from distant phyla to identify critical metabolic pathways in *C. burnetii*.

(2) Because the intracellular growth of *C. burnetii* within THP-1 cells is not as high as in Vero cells, we might not recover enough bacterial mRNA to accurately analyze gene expression. If that is the case, we will sequence without multiplexing, which should provide enough depth to quantify gene expression.

(3) Both *C. burnetii* RSA 493 Nine Mile phase I and phase II strains have been shown to have similar growth kinetics within human macrophages (Shannon and Heinzen 2008). However, because the phase II strain is an LPS mutant that is not infective to humans, it is possible that the intracellular metabolic activity of this strain is slightly different from that of the phase I strain. To confirm that gene expression in phase II and phase I strains are similar, Dr. James Samuel will provide us with RNA extracted from THP-1 cells infected with Phase I *C. burnetii* in his BSL-3 facility (see attached Letter of Support). We will analyze phase I RNA samples using RNA-seq, as described above for phase II *C. burnetii*.

Specific Aim 3. Determine the importance of heme biosynthesis to *C. burnetii's* intracellular growth.

Background and Justification

In order to validate our novel approach to identifying critical metabolic processes in *Coxiella,* we will examine the importance of heme biosynthesis to *Coxiella*'s intracellular growth. This biosynthetic pathway was chosen because it is unique to the human pathogen, contains horizontally derived genes, and is expressed intracellularly, i.e. this is an example of a pathway that we expect to prioritize after concluding Aims 1 and 2.

Coxiella's genome is less than half the size of *E. coli*'s genome (~2 Mb and ~5 Mb, respectively). Correspondingly, *Coxiella* has less than half the number of tRNAs than in *E. coli* (42 and 89, respectively). This reduction has occurred due to loss of redundant tRNAs. For instance, while *E. coli* has five copies of $tRNA^{Ile}$, *Coxiella* has only one copy. Against this constant culling of redundant tRNAs, *Coxiella* has acquired, via HGT, an additional $tRNA^{Glu}$ isoacceptor ($tRNA^{Glu}2$, anticodon CUC) that is not found in any other Gammaproteobacteria (**Figure 9**). Because $tRNA^{Glu}2$ is retained in such a streamlined genome, and is expressed at very high amounts (double that of the other $tRNA^{Glu}$ isoacceptor, $tRNA^{Glu}1$, anticodon UUC) during intracellular growth (**Figure 10**), it most likely has a critical contribution to bacterial fitness.

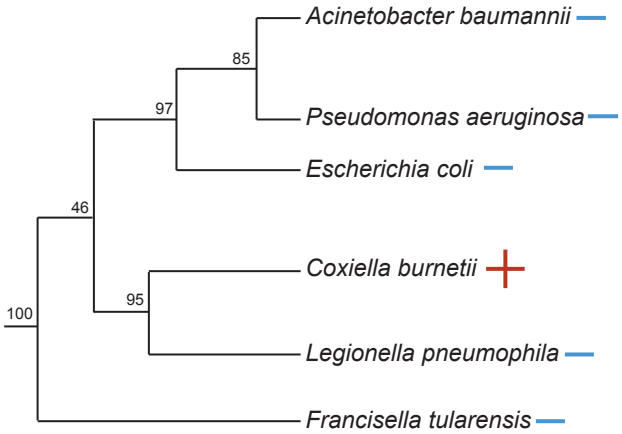


Figure 9. A 16S phylogenetic tree of Gammaproteobacteria showing the presence (+) or absence (-) of $tRNA^{Glu}2$.

Intriguingly, the major function of $tRNA^{Glu}2$ is unlikely to be protein synthesis. There are two codons for glutamic acid: GAA and GAG. In *Coxiella* proteins, 76% of glutamate codons are GAA, which $tRNA^{Glu}2$ (anticodon CUC) cannot decode, whereas $tRNA^{Glu}1$ (anticodon UUC) can decode both codons efficiently. Previous studies have shown elevated protein synthesis in *Coxiella* when incubated in a glutamate-supplemented medium (Hackstadt and Williams 1983; Omsland et al. 2008). To rule out the possibility that *Coxiella* proteins were enriched for glutamate, and hence needed the extra $tRNA^{Glu}$ for efficient translation, we determined the total number

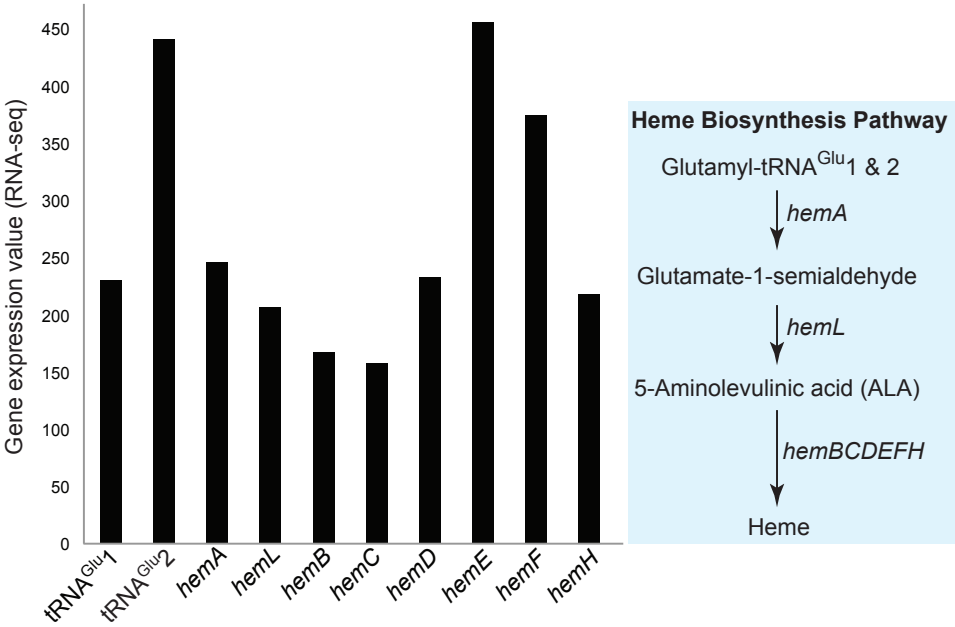


Figure 10. Expression of $tRNA^{Glu}2$ and heme biosynthesis genes within Vero cells.

of Glu codons in *Coxiella* genes and compared it to *E. coli* genes. We found that both *E. coli* and *Coxiella* proteins have similar Glu content (~6% of all codons). Together, these data suggest that protein biosynthesis is not $tRNA^{Glu}2$'s major function in *C. burnetii*.

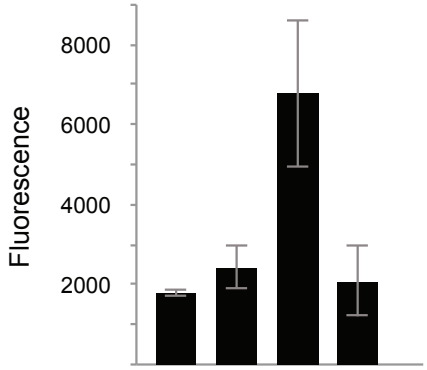
Heme is an iron-containing tetrapyrrole that serves multiple cellular functions including in respiration, energy generation, oxidative reactions and signal transduction (Almiron et al. 2001). The universal precursor of tetrapyrrole biosynthesis is 5-aminolevulinic acid (ALA). There are two alternate pathways in nature for the synthesis of ALA: C5 pathway found in most bacteria, and Shemin pathway present in most eukaryotes, including humans (Frankenberg et al. 2003). The starting point of the C5 pathway is Glutamyl-$tRNA^{Glu}$, which is converted into ALA using two consecutive enzymes HemA and HemL (**Figure 10**, previous page). In Shemin pathway ALA is synthesized from Succinyl-CoA and glycine by the enzyme ALAS (Frankenberg et al. 2003). The rest of the steps are shared between C5 and Shemin pathways. We examined the *C. burnetii* genome and discovered that it contains an intact C5 heme biosynthesis pathway, and by RNA-seq we confirmed that these genes are utilized during growth within Vero cells (**Figure 10**, previous page). This pathway is conserved in all *C. burnetii* strains but is absent in non-pathogenic *Coxiella* present in ticks (**Table 3**), indicating its importance to the human pathogen. Additionally, a Tn-insertion in the 3' end of *hemL* caused significant growth inhibition (see **Figure 8**, Aim 2). Based on these data, our *working hypothesis* is that the major function of $tRNA^{Glu}2$ is as a substrate for heme biosynthesis, which is crucial to *C. burnetii*'s intracellular growth.

Table 3. Heme biosynthesis genes are conserved in all *C. burnetii* (blue) but not in CLEAA seen in *Amblyomma americanum* and CRt in *Rhipicephalus turanicus* (red).

	RSA 493	RSA 331	DUG-WAY	Q212	Q154	Z3035	CLEAA	CRt
hemA	+	+	+	+	+	+	–	–
hemL	+	+	+	+	+	+	–	–
hemB	+	+	+	+	+	+	–	–
hemC	+	+	+	+	+	+	–	–
hemD	+	+	+	+	+	+	–	–
hemE	+	+	+	+	+	+	–	–
hemF	+	+	+	+	+	+	–	–
hemH	+	+	+	+	+	+	–	–
$tRNA^{Glu}2$	+	+	+	+	+	+	–	–

Preliminary Studies and Feasibility

1. *C. burnetii* cannot use extracellular heme as an iron source. *C. burnetii* encodes a transporter system (*feoAB*) for ferrous iron, which is required for heme biosynthesis, but has no known transporters for ferric ions or heme. Consequently, we reasoned that the pathogen might not be able to utilize extracellular heme as its sole source of iron. To test this, we inoculated equal amounts of *Coxiella* into ACCM-2 medium with $FeSO_4$ (standard recipe) or into ACCM-2 in which $FeSO_4$ was replaced with equimolar amount of hemin. Growth was measured at 7 d pi using PicoGreen as described previously (Martinez et al. 2015). As shown in **Figure 11**, *Coxiella* growth in $FeSO_4$-containing ACCM-2 was significantly higher than in hemin-containing ACCM-2 ($p < 0.001$, paired t-test), whereas the fluorescence measurements between control samples (ACCM-2 only) and the hemin-containing ACCM-2 samples were not significantly different ($p > 0.05$, paired t-test), indicating that *C. burnetii* cannot utilize external heme.



C. burnetii	–	+	+	+
Heme	–	+	–	–
$FeSO_4$	+	–	+	+
Gabaculine (1 mM)	–	–	–	+

Figure 11. Hemin in place of $FeSo_4$, and 1 mM Gabaculine caused significant growth inhibition (n=4).

2. Gabaculine inhibits *C. burnetii* growth. To test *C. burnetii*'s requirement for heme biosynthesis, we treated *C. burnetii* with increasing concentrations of gabaculine, an inhibitor of HemL (Wang et al. 1997). As shown in **Figure 11**, 1 mM gabaculine significantly inhibited the growth of *C. burnetii* ($p < 0.001$, paired t-test), confirming the importance of heme biosynthesis to its growth. We were unable to test gabaculine against *C. burnetii* growing in host cells because the drug is toxic to mammalian cells. As a follow up to this project, we plan to screen small molecule libraries to identify novel chemicals that block *C. burnetii*'s heme biosynthesis without causing toxicity in human cells (see Future Directions).

3. Generation of $tRNA^{Glu}2$-deletion strain. To assay the importance of $tRNA^{Glu}2$, we generated a $tRNA^{Glu}2$-deletion strain. This was accomplished in collaboration with Dr. Paul Beare at Rocky Mountain Laboratories who developed the *Coxiella* genetic system (Beare et al. 2012) (see Letter of Support). Briefly, *C. burnetii* was transformed with a plasmid (pJC-kan-Glu2-CAT) and a clonal population in which $tRNA^{Glu}2$ was replaced by Chloramphenicol acetyltransferase (CAT)

gene was isolated as described previously (Beare et al. 2012) (**Figure 12**).

Research Design

1. Complementation of tRNA^{Glu}2-deletion strain. In order to confirm that phenotypes displayed by the tRNA^{Glu}2-deletion strain are not due to aberrant changes, we will construct a complementation strain. Briefly, the tRNA^{Glu}2-deletion strain will be transformed with a plasmid pTn7-tRNAGlu2, which encodes a transposase that will reintroduce tRNA^{Glu}2 along with its promoter into the intergenic region between CBU_1787 and CBU_1788, as described previously (Beare et al. 2011; Beare et al. 2012). As with the construction of the deletion strain, we will collaborate with Dr. Paul Beare to generate the complementation strain (see Letter of Support).

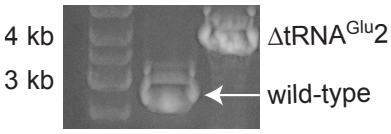


Figure 12. PCR gel confirming chloramphenicol resistance gene (CAT) replaced tRNAGlu2.

2. Quantification of heme production. Heme content of wild-type, tRNA^{Glu}2-deletion, and complementation strains of *Coxiella* grown in ACCM-2 will be quantified as described previously (Hughes and Macvanin 2010). Briefly, *Coxiella* cells will be collected by centrifugation and washed in phosphate-buffered saline. A 50 μl aliquot will be mixed with 500 μl of heated 2 M oxalic acid and incubated at 100°C for 30 min. This treatment strips iron from heme and the remaining protoporphyrin can be measured by fluorescence. After 30 min, 500 μl of phosphate buffer will be added to the mixture and allowed to cool, and the fluorescence spectra of the samples will be measured.

3. Quantification of intracellular growth rate. THP-1 cells differentiated into an adherent, macrophage-like morphology will be infected with SCVs of wild-type, tRNA^{Glu}2-deletion and complementation strains of *C. burnetii* at an MOI of 25, as described in Aim 2. We will collect THP-1 cells every 24 h, disrupt cell pellets using a bead-beater (Bio-Spec) and Phenol:Chloroform (Life technologies) and isolate DNA using a Qiagen DNA extraction kit. Quantitative PCR (qPCR) using primers specific to *rpoS* and *dotA* genes will be used to measure intracellular *Coxiella* as described previously (Raghavan et al 2008; Omsland et al. 2011). In parallel to qPCR, we will quantify intracellular *Coxiella* using florescence microscopy. We will transform wild-type, tRNA^{Glu}2-deletion and complementation strains with the vector pKM244 that expresses the mCherry red florescent protein (Chen et al. 2010). Parasitophorous vacuoles formed within THP-1 cells by *C. burnetii* strains will be enumerated by florescence imaging, as described previously (Chen et al. 2010; Beare et al. 2011; Martinez et al. 2014). To prepare for this project, we have procured the plasmid pKM244 from Dr. Samuel, and we have a Leica TCS SPE confocal fluorescence microscope.

Expected Outcomes

(1) We expect both heme production and intracellular growth of the tRNA^{Glu}2-deletion strain to be significantly lower than that of the wild-type strain, and expect both measures to be restored in the complementation strain. These data will validate the importance of heme biosynthesis to *C. burnetii*'s intracellular growth, which will be a <u>*significant advancement*</u> in the field because this will be the first metabolic pathway that will be shown to be critical *C. burnetii*'s intracellular growth. Moreover, we will validate heme biosynthesis as a promising a target for developing new therapeutic agents to treat chronic Q fever, which is the next step in accomplishing our long-term goal (see Future Directions).

(2) We expect the horizontally derived tRNA^{Glu}2 to improve the pathogen's heme production by making available more charged tRNA^{Glu} for heme biosynthesis. Having this extra tRNA^{Glu} is expected to free up enough tRNA^{Glu} for protein synthesis so that heme production will not impinge upon bacterial growth. This discovery will reveal a <u>*novel pathogenic mechanism*</u> in bacteria whereby an alternate tRNA promotes virulence. Just as in *Coxiella*, several important human pathogens such as *Bacteroides, Corynebacterium*, and *Clostridium* encode multiple tRNA^{Glu} isoacceptors, and hence might be utilizing a similar pathogenic strategy. Our approach of using genome evolution to identify virulence processes and potential antibiotic targets could be applied broadly to other pathogens to combat rising antibiotic resistance.

(3) Student training. Abraham Moses, who is currently a Research Associate, produced most of the preliminary data described in this Aim while he was working as an undergraduate student in the lab. He will perform all the experiments described in this Aim. He plans to join our graduate program in couple of years. Todd Hinsch, an undergraduate student in the lab will work with Abraham and me to accomplish the goals of this Aim. After graduation, he plans to join the Molecular Microbiology graduate program at OHSU.

Potential Problems and Alternative Strategies

(1) If the heme quantification method described above does not produce consistent results, we will utilize an alternate approach described by Levican et al. (2007). In this method, *Coxiella* cells will be washed in 30 mM Hepes, pH 8.0 and suspended in a solution containing 0.5 M NaOH and 2.5% Triton-X. Cells will be broken up using sonication and centrifugation, and heme content will be measured at 575 nm absorbance. Another alternative is a commercially available Heme Assay Kit (Abnova).

(2) If heme production or intracellular growth (or both) is not impacted by the loss of $tRNA^{Glu}2$, we will compare the proteomes of wild-type and $tRNA^{Glu}2$ deletion strains. We have prior experience with *Coxiella* proteomics (Raghavan et al. 2008; Hicks et al. 2010), and the OHSU Proteomics Shared Resources has the staff and equipment required for successful proteome analyses of *Coxiella* strains. Briefly, we will grow *C. burnetii* strains in ACCM-2 for 7 days and the bacteria will be pelleted by centrifugation (20,000 x g for 5 min). Proteins from a total cell lysate will be prepared as described earlier (Hicks et al. 2010; Stead et al. 2013). Proteins will be separated by SDS-PAGE using 16.5% gel and visualized using SimplyBlue SafeStain (Invitrogen), and the lanes will be cut into 10 equal sections and subjected to trypsin digestion. The resulting peptides will be sequenced by tandem mass spectrometry (MS/MS) at the OHSU Proteomics Shared Resources using a ThermoFinnigan LTQ mass spectrometer fitted with an IonMax nanospray source. The MS/MS spectra will be searched against *C. burnetii* subset of NCBI nr protein database using Proteome Software Scaffold 3.0. The proteomic data is expected to reveal the proteins that were impacted by the loss of $tRNA^{Glu}2$. Genes that encode these proteins will then be interrogated to identify metabolic processes that are important to *Coxiella*'s intracellular growth and virulence.

(3) Because *C. burnetii* contains two $tRNA^{Glu}$ isoacceptors, it is possible that the lack of $tRNA^{Glu}2$ will not have any impact on the pathogen. However, this is not a concern because in comparison to wild-type *C. burnetii*, the $tRNA^{Glu}2$-deletion strain exhibited a severe growth defect in ACCM-2 (**Figure 13**), indicating that $tRNA^{Glu}2$ is essential for attaining optimum fitness, most likely through augmenting heme production.

(D) FUTURE DIRECTIONS

At the successful completion of this project, we expect to gain new insights into the evolution of *C. burnetii*, and to have identified metabolic pathways that are critical to its intracellular growth. Our next steps will be:

(1) As in Aim 3, we will delete specific genes to validate the importance of *C. burnetii*'s metabolic pathways. For example, we have evidence that biotin biosynthesis is also critical for *C. burnetii*'s growth. No transposon-insertion mutants are currently available for biotin biosynthesis genes, but we expect to be able to generate a *C. burnetii* strain lacking biotin biosynthesis by providing the cofactor in ACCM-2 medium, as shown recently for other nutrients (Sandoz et al. 2016).

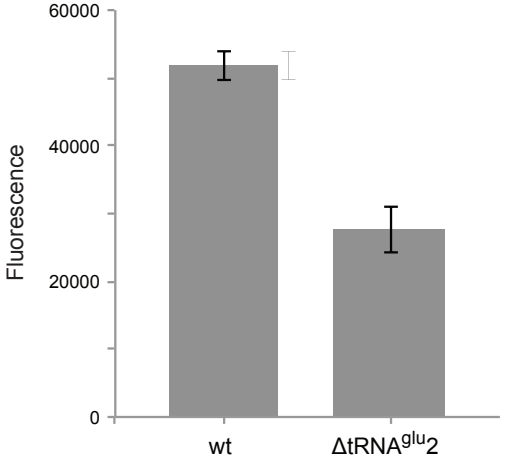
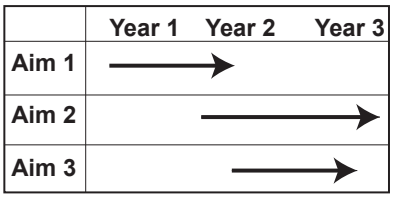


Figure 13. *C. burnetii* growth in ACCM-2 measured at 7 d post-inoculation using PicoGreen. $p < 0.001$ (paired t-test, n = 3).

(2) The next step towards achieving our long-term goal of developing new therapeutic agents will be to identify chemical agents that inhibit the function of critical metabolic genes. For instance, the small molecule MAC13772 (Maybridge) inhibits biotin biosynthesis in *E. coli* (Zlitni et al. 2013). To identify small molecules that block critical metabolic processes in *C. burnetii*, we will conduct screens using small molecule libraries. In collaboration with Dr. Robert Allen (see Letter of Support) at Oregon Translational Research and Development Institute (OTRADI), Austin Wright, a first-year graduate student in the lab will perform these experiments.

(E) RESEARCH TIMELINE.

This project will take three years to complete.



SELECT AGENTS

The proposed research involves *Coxiella burnetii*, a Category B Select Agent. However, all work will use the avirulent, exempt Nine Mile Phase II strain (RSA 439), under BSL2 containment. Genes conferring resistance to kanamycin and chloramphenicol are approved for *Coxiella burnetii* genetic transformation by the Centers for Disease Control and Prevention Select Agents Program.

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