CURRICULUM VITAE

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SUMMARY OF QUALIFICATIONS

- Extensive experience studying infectious disease and virulence mechanisms of a variety of BSL-2 and BLS-3 bacterial pathogens
- Expertise with bacterial culture, intracellular bacteria, cell culture, transfection and transformation, state of the art cloning techniques and recombinant proteins
- Published author knowledgeable in characterization of bacterial lipids and proteins
- Teaching experience at the university level
- Excellent proficiency in working independently and in multi-disciplinary/multicultural research teams
- Excellent written and oral communication skills with both scientific and non-scientific audiences

EDUCATION

Louisiana State University, Department of Biological Sciences, Class of 2010
Bachelor of Science, Biology (Faculty mentor: Kevin R. Macaluso, Ph.D.)
Baton Rouge, Louisiana

The College of William and Mary, Department of Biology, Class of 2013
Master of Science, Biology (Faculty mentor: Oliver Kerscher, Ph.D.)

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July 2018
Ph.D., Microbiology and Immunology (Faculty mentor: Abdu Azad, Pharm.D., Ph.D.)

WORK EXPERIENCE

1. **Graduate Research Assistant, T32 Training Grant in Innate Immunity. Graduate Program in Life Sciences, Department of Molecular Microbiology and Immunology, University of Maryland Baltimore, Baltimore, MD. Lab of Abdu Azad,** September 2013 – Present.
   - ***Thesis project*:**
     - BSL-2, BSL-3 work with *Rickettsia typhi, R. rickettsii*, and non-pathogenic rickettsiae characterizing the structure and synthesis of the lipid A moiety of lipopolysaccharide. I Investigated lipid A diversity between species of *Rickettsia*, identified a novel lipid A structure in the highly virulent *R. rickettsii* str, Sheila Smith, and characterized two novel members of the

recently described LpxJ family of acyltransferases of the lipid A biosynthetic pathway.

2. <u>**Graduate Teaching Assistant,**</u> **Masters of Science Program in Biology, Department of Biology, The College of William and Mary, Williamsburg, Virginia. Lab of Oliver Kerscher,** Autumn 2011 – Summer 2013.
   - ***Thesis project*:**
     - Cell division cycle dynamics and protein turnover controlled by SUMOylation and SUMO-targeted ubiquitin ligases in budding yeast, *Saccharomyces cerevisiae*. I investigated the E3 SUMO ligase, Siz1, and its SUMOylation substrates as they interact with a SUMO-targeted ubiquitin ligase known as Slx5.

3. <u>**Research Assistant,**</u> **Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana. Lab of Kevin Macaluso,** Spring 2010 – Autumn 2011.
   - ***Research project*:**
     - BSL-2, aBSL-2 work with *Rickettsia felis* as it interacts with its insect vector, *Ctenocephalides felis* (Cat flea), and a recently described symbiotic host, *Liposcelis bostrychophila* (Book louse). I was responsible for maintenance of two colonies of cat fleas, including one that was maintained on mammalian hosts. Additionally, I began a new colony of book louse (LSU colony) from scratch and assisted with the isolation of a new strain of *R. felis* (*R. felis* LSU-Lb). My project included investigating transcriptional differences between flea- and louse-associated *R. felis* strains, as well as hunting for a possible route of transmission of *R. felis* between the two arthropods hosts.

PUBLICATIONS

Guillotte M.L., Chandler C.E., Gillespie J.J., Yoon S.H., Rahman M.S., Ernst R.K., Azad AF. (2018 in prep). <u>Structural features of lipid A of *Rickettsia rickettsii*.</u>
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Rennoll, S. A., Rennoll-Bankert, K. E., **Guillotte, M. L.**, Lehman, S. S., Driscoll, T. P., Beier-Sexton, M., … Azad, A. F. (2018). <u>The cat flea (Ctenocephalides felis) immune deficiency signaling pathway regulates *Rickettsia typhi* infection</u>. *Infection and Immunity*, *86*(1). http://doi.org/10.1128/IAI.00562-17
Driscoll, T. P., Verhoeve, V. I., **Guillotte, M. L.**, Lehman, S. S., Rennoll, S. A., Beier-Sexton, M., … Gillespie, J. J. (2017). <u>Wholly *Rickettsia* ! Reconstructed Metabolic Profile of the Quintessential Bacterial Parasite of Eukaryotic Cells</u>. *mBio*, *8*(5), e00859-17. http://doi.org/10.1128/mBio.00859-17
Gillespie, J. J., Phan, I. Q. H., Driscoll, T. P., **Guillotte, M. L.**, Lehman, S. S., Rennoll-

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Petchampai, N., Sunyakumthorn, P., **Guillotte, M. L.**, Thepparit, C., Kearney, M. T., Mulenga, A., … Macaluso, K. R. (2014). Molecular and functional characterization of vacuolar-ATPase from the American dog tick *Dermacentor variabilis*. *Insect Molecular Biology*, *23*(1), 42–51. http://doi.org/10.1111/imb.12059
Petchampai, N., Sunyakumthorn, P., **Guillotte, M. L.**, Verhoeve, V. I., Banajee, K. H., Kearney, M. T., & Macaluso, K. R. (2014). Novel Identification of Dermacentor variabilis Arp2/3 Complex and Its Role in Rickettsial Infection of the Arthropod Vector. *PLoS ONE*, *9*(4), e93768. http://doi.org/10.1371/journal.pone.0093768
Thepparit, C., Sunyakumthorn, P., **Guillotte, M. L.**, Popov, V. L., Foil, L. D., & Macaluso, K. R. (2011). Isolation of a Rickettsial Pathogen from a Non-Hematophagous Arthropod. *PLoS ONE*, *6*(1), e16396. http://doi.org/10.1371/journal.pone.0016396

RESEARCH ABSTRACT PRESENTATIONS

1. Oral Presentation: UMB/UMCP Cross-Talk Symposium; Rockville, MD, June 2017
   Title: Insight into the form and function of rickettsial Lipid A

2. Poster Presentation: American Society for Microbiology Maryland Branch; Baltimore, MD, May 2017
   Title: Structural Characterization of Lipid A of *Rickettsia*

3. Poster Presentation: Cold Spring Harbor Microbial Pathogenesis & Host Response; Cold Spring Harbor, New York, September 2017
   Title: Insight into the form and function of rickettsial lipid A

4. Poster Presentation: International Endotoxin and Innate Immune Society; Hamburg, Germany, September 2016
   Title: Structural Characterization of Lipid A of *Rickettsia*
5. Poster Presentation: American Society of Rickettsiology annual meeting; Lake Tahoe, CA, June 2015
   Title: Insect Innate Immune Response to *Rickettsia typhi*
6. Poster Presentation: American Society of Cell Biology annual meeting; San Francisco, CA, December 2012
   Title: SUMO-Targeted Ubiquitin Ligase-mediated degradation of Nuclear Siz1

LABORATORY SKILLS AND TECHNIQUES

- **Laboratory Safety Levels:**
  - **MARCE Certification**: trained in BSL-3 laboratory safety, University of Maryland School of Medicine Mid-Atlantic Regional Center of Excellence BSL-3 training course (2014), plus annual BSL-3 training refreshers (UMB Environmental Health and Safety training)
- **General Microbiology:**
  Sterile technique; preparation of selective media; DNA purification of plasmid and chromosomal DNA; Protein purification; Culture and purification of *Rickettsia* species; growth and purification of *S. cerevisiae* and *E. coli*. General tissue culture maintenance.
- **Molecular Techniques:**
  Cloning; gel electrophoresis; extraction of DNA out of agarose gel; editing and analyzing plasmid maps (multiple software types); sequencing and interpreting nucleotide sequencing data; designing and ordering primers; PCR; site specific mutagenesis of plasmid DNA, RT-qPCR (and ΔΔCt analysis)
- **Genetic Techniques:**
  Transfection, chemical transformation; conjugation; electroporation; Cre-lox system, theophylline induced aptamer-based gene expression
- **Arthropod Techniques:**
  Maintenance and propagation of flea colony (*Ctenocephalides felis*), siRNA feeding, microscopy of infected flea sections. Maintenance and propagation of book louse colony (*Liposcelis bostrychophila*)
- **Immunological Techniques:**
  Toll-like receptor reporter assay. Culture of mouse macrophages, cytokine analysis (ELISA)
- **Microscopy Techniques:**
  Immunofluorescence microscopy, confocal microscopy (Zeiss LSM 510 Meta)
- **Protein Analysis Techniques:**
  SDS-PAGE, Western blotting, densitometry, hybrid-protein (tagged) construction, affinity column protein isolation, pull-down, co-immunoprecipitation, large-scale protein purification for crystallization studies
- **Lipid analysis and Mass spectrometry Techniques:**
  Extraction of lipid A from *E. coli* and *Rickettsia* (Grown in tissue culture) using several methods; MS-based lipid analysis on a Bruker Microflex series and Autoflex series MALDI-TOF systems.
- **Mammalian Cell Culture Techniques:**
  Standard cell culture of HeLa, Vero76, HEK293T, THP-1, RAW265.7, A431, ISE6

AWARDS AND FELLOWSHIPS

1. Arts & Science OGSR/Graduate Student Association Conference Fund, College of William & Mary, 2012

2. Travel Award – 14th biennial meeting; International Endotoxin and Innate

Immunity Society

3. Institutional National Research Service Award (T32; **T32AI095190**) to University of Maryland Baltimore - Signaling Pathways in Innate Immunity (Pre-doctoral trainee) 2015-present

TEACHING EXPERIENCE

- **Graduate Teaching Assistant**: Microbiology Laboratory, The College of William and Mary
  - Spring semester 2013

- **Graduate Teaching Assistant**: Introduction to Molecules, Cells, and Development, The College of William and Mary
  - Spring semester 2012

- **Graduate Teaching Assistant**: Introduction to Organisms, Ecology, and Evolution, The College of William and Mary
  - Fall semester 2011, 2012

PROFESSIONAL AFFILIATIONS

1. Student member of American Society of Cell Biology, 2012-2014

2. Student member of American Society of Microbiology, 2014-present

3. Student member of American Society of Rickettsiology, 2014-present

4. Student member of the International Endotoxin and Innate Immunity Society, 2016-present

PROFESSIONAL AFFILIATIONS

- Student employee, August 2006 to May 2010; Vector Biology Laboratory, Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University

- Volunteering for new student recruitment

- Assist training of Postbaccalaureate and M.Sc. students
  - Proma Ahmed (BS, Virginia Tech University 2016)
  - Chien-Ju Chien (M.Sc., University of Maryland Baltimore 2017)

Abstract

Dissertation title: Structure and synthesis of Lipid A of *Rickettsia* species

Mark L Guillotte, Doctor of Philosophy, 2018

Dissertation Directed By: Abdu Azad, PharmD, PhD, MPH

Members of the *Rickettsia* genus are obligate intracellular, Gram-negative coccobacilli that infect mammalian and arthropod hosts. Several rickettsial species are human pathogens and are transmitted by blood-feeding arthropods. In mouse infection models, control of rickettsial burden and disease resolution depends upon inflammatory cytokine production that is driven, in part, by Toll-like receptor (TLR) activation. The lipid A component of Gram-negative lipopolysaccharide (LPS) is the classical agonist of TLR4, however lipid A structure of rickettsial LPS and its inflammatory potential are unknown. Here we report the structure of lipid A from several species of *Rickettsia* including fatty acid analysis of *Rickettsia rickettsii* str. Sheila Smith, the most virulent species and the etiological agent of Rocky Mountain Spotted Fever. Furthermore, we have identified and characterized a new member of the recently discovered LpxJ family, a late-acyltransferase in *Rickettsia typhi*, the etiological agent of murine typhus, and *R. rickettsii*. Our results demonstrate that this enzyme, LpxJ, catalyzes the addition of a secondary acyl chain (C16) to the 3'-linked primary acyl chain of the lipid A moiety in the last step of the Raetz pathway of lipid A biosynthesis. Since lipid A architecture is fundamental to bacterial outer-membrane integrity, we believe LpxJ is important in maintaining ideal membrane dynamics to facilitate molecular interactions at the host-pathogen interface that are required for adhesion and invasion of

mammalian cells. This work promises to reveal novel insights into Rickettsia pathogenesis and contribute greatly to our understanding of rickettsial physiology.

STRUCTURE AND SYNTHESIS OF LIPID A OF *RICKETTSIA* SPECIES

by
Mark L. Guillotte

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2018

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DEDICATION

This dissertation is dedicated to the life and memory of Professor Mark E. Shirtliff. He was an exceptional person, a great friend, and the best teacher I’ve ever had. As a university family, our lives will be less for his absence.

ACKNOWLEDGEMENTS

I am forever grateful to my parents, Tammy and Calvin, and to my family for the opportunities and support they have provided to me throughout my life. While growing up my family always encouraged me to be curious, creative, and thoughtful and this has made me into the person and scientist that I am today. Thank you to my wife Jessica for getting me through the daily struggles of graduate school and for being there for me when I was feeling overwhelmed. I truly couldn't have done it without her, and I would never want to. The most important honor that I have ever been given is knowing that my family is proud of me.

Dr. Kevin Macaluso is the reason I am in graduate school. As my first mentor, it was in his laboratory that I learned what it meant to be a scientist. His drive and passion for research was the perfect example for a young student, which fostered in me a need to better myself and an ambition to make a difference in the world, the same way he does. Thank you for taking in a floundering teenager. I am a better person for knowing you.

This dissertation would not have been possible without the support of my academic advisor, mentor, and friend Dr. Abdu F. Azad. Under his guidance I have developed into an independent scientist and, through his support, I have been able to pursue my scientific interest in my PhD studies, culminating in the body of work detailed herein. I am eternally grateful for his support and I hope one day I can replicate his success in my own lab.

I am grateful to Drs. Joe Gillespie and Sayeed Rahman. During my graduate studies, their help and advice have guided my scientific endeavors and facilitated my success as both a student and as a person. I cannot speak highly enough of their generosity and passion for science, as well as their abilities as teachers, mentors, and friends. Thank you, and I will miss our lunch-time adventures as well as our discussions over coffee; the ones about science and about politics.

I am also proud to call all of my lab mates my friends, especially Magda Beier-Sexton for her generous support of myself, this research project, and the lab. Additionally, thank you to Dr. Kristen Rennoll-Bankert, my first teacher in the Azad lab, for her kindness and patience.

Thank you to Stephanie Lehman, my fellow graduate student in the Azad lab. She was always willing to share a kind word and a cold beer after a tough week. I wish my lab sister all the happiness and success in the world.

I would like to thank Dr. Robert Ernst for his willingness to collaborate with Dr. Azad and I on my project, and sometimes, for tolerating me taking up space and reagents in his laboratory. He is an exceptional professor and a good friend who is always ready and willing to help other before himself. These aspects of his character are evident when you consider the people surrounding him in the Ernst lab. The technical assistance, moral support, and jokes from members of the Ernst lab have been indispensible in supporting this project and my sanity. To Courtney Chandler, Francesca Gardner, Kelsey Gregg, Erin Harberts, Belita Opene, Alison Scott, and Sung Hwan Yoon – Thank you for everything!

I am grateful to Dr. Stephanie Vogel for her kindness, advice, wisdom, and most importantly, her friendship. Her thoughtful mentorship has made me a better scientist, student, and person.

Finally, thank you to my committee members Dr. Eileen Barry, and Dr. Bret Hassel, whose teaching, constructive criticism, and advice in our meetings and elsewhere have been an essential to my research and my success as a student.

The Graduate Program in Life Sciences and the Department of Microbiology and Immunology are a family, and I have made life-long friends throughout my PhD program. I look forward to a lifetime of professional and social collaboration with all of you. As in any job, there were highs and lows. However, I can say with sincerity that I wouldn’t trade it for the world; if I could do it all over again, I would do it the same way.

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LIST OF ABBREVIATIONS

Arp2/3, Actin related proteins 2/3
ATF2, Activating transcription factor 2
BAME, Bacterial fatty acid methyl esters
C;M, Chloroform:methanol ratio
C#, Fatty acid of # length of carbons
Casp, Caspase
CXCL, Cysteine-x-cysteine motif ligand (chemokine)
CD, Cluster of differentiation
DHAPAT, Glyceronephosphate O-acyltransferase
DMEM, Dulbecco's modified Eagle's medium
DNA, Deoxyribonuleic acid
DUF, Domain of unknoen function
FBS, Fetal bovine serum
Gln, Glucosamine
GPAT, Glycerol-3-phosphate acyltransferase
hr, Hour(s)
IPTG, Isopropyl β-D-1-thiogalactopyranoside
KDO, 3-Deoxy-D-manno-oct-2-ulosonic acid
LB, Luria broth
LPAAT, Lysophosphatidic acid acyltransferase
LPEAT, Lysophosphatidylethanolamine acyltransferase
LPS, Lipopolysaccharide
MALDI-TOF, Matrix-assisted laser desorption/ionization-time of flight
MAPK, Mitogen activated protein kinase
Mb, Megabase
MD2, Lymphocyte antigen 96
MOI, Multiplicity of infection
MS, Mass spectrometry
OM, Outer membrane
OMP, Outer membrane protein
p.i., Post infection
PAMP, Pathogen associated molecular pattern
PBS, Phosphate buffered saline
PCR, Polymerase chain reaction
PRR, Pattern recognition receptor
RMSF, Rocky Mountain spotted fever
RNA, Ribonucleic acid
rRNA, Ribosomal ribonucleic acid
RT-PCR, Reverse transcriptase polymerase chain reaction
RT-qPCR, Reverse transcription quantitative polymerase

chain reaction

Rt, *R. typhi*

Sca, Surface cellular antigen

SDS, Sodium dodecyl sulfate

Sec, Seconds

SFG, Spotted fever group

SS, Sheila Smith

STAT, Signal transducer and activator of transcription

TG, Typhus group

TLR, Toll-like receptor

TRG, Transitional group

WT, Wild type

CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 Overview and history

Members of genus *Rickettsia* are obligate intracellular Gran-negative bacterial parasites of Eukaryotes. First described in the Bitterroot valley of Montana in 1906 (1, 2), *Rickettsia* are small (0.3-0.5x0.8-2.0µm) coccobacilli that have adapted to live and replicate within the eukaryotic cytosol. As a genus, *Rickettsia* is comprised of 27 named species, of which 17 are known or suspected pathogens (Table 1.1) (3). Historically, the genus has been split into two groups, Spotted Fever group and Typhus group, based on serology and disease pathology. The molecular revolution has brought with it an increasing number of sequenced genomes from which estimated phylogenies have driven the establishment of two additional groups, Ancestral group and Transitional group (4). Pathogenic members are spread to humans via hematophagous arthropods such as fleas, ticks, lice, and mites (5). The most virulent species include the etiological agents of epidemic typhus (*R.* prowazekii) and Rocky Mountain Spotted Fever (*R. rickettsii*), which are among the most lethal human pathogens (6).

Table 1.1: Named species, phylogenetic grouping, and known disease association

Organism	Group	Disease
R. rickettsii	Spotted Fever Group	Rocky Mountain spotted fever
R. prowazekii	Typhus Group	Epidemic typhus
R. conorii	Spotted Fever Group	Mediterranean spotted fever
R. typhi	Typhus Group	Murine typhus
R. sibirica	Spotted Fever Group	Siberian tick typhus
R. australis	Transitional Group	Queensland tick typhus
R. akari	Transitional Group	Rickettsialpox
R. slovaca	Spotted Fever Group	Tick-born lymphadenopathy
R. parkeri	Spotted Fever Group	Maculatum disease
R. japonica	Spotted Fever Group	Japanese spotted fever
R. honei	Spotted Fever Group	Flinders Island spotted fever
R. africae	Spotted Fever Group	African tick bite fever
R. massiliae	Spotted Fever Group	Spotted fever rickettsiosis
R. aeschlimanii	Spotted Fever Group	Spotted fever rickettsiosis
R. heilongjiangensis	Spotted Fever Group	Far Eastern spotted fever
R. monacensis	Spotted Fever Group	Spotted fever rickettsiosis
R. helvetica	Spotted Fever Group	Spotted fever rickettsiosis
R. felis	Transitional Group	Flea-borne spotted fever
R. raoulti	Spotted Fever Group	
R. asiatica	Spotted Fever Group	
R. bellii	Ancestral Group	
R. buchneri	Spotted Fever Group	
R. canadensis	Ancestral Group	
R. hoogstraalii	Transitional Group	
R. montanensis	Spotted Fever Group	
R. peacockii	Spotted Fever Group	
R. rhipicephali	Spotted Fever Group	
R. tamurae	Spotted Fever Group	
R. amblyommatis	Spotted Fever Group	

Rickettsia species are maintained generationally in their arthropod host principally through transovarial and transstadial transmission. A low rate of horizontal transmission occurs when an uninfected arthropod feeds on an infected vertebrate host, replenishing or expanding the reservoir population (3). As obligate parasites, the prevalence and distribution of rickettsiae depends on the abundance and habitat range of their reservoir arthropods. Globalization and climate change have increased the threat of tropical disease due, in part, to expansion of habitat range of disease vectors (7). As these trends continue, *Rickettsia* species will present an increasing public health risk to humans and other animals. Ecological countermeasures and therapeutic interventions mitigating the expanse of global rickettsial burden require a thorough understanding of the molecular and immunological determinants of both human disease as well as vector competence.

1.2 Rickettsial diseases

Human infections caused by species of *Rickettsia* are diverse in their severity, ranging from mild, nearly asymptomatic infections caused by newly described pathogens (8), to life-threatening diseases that require intense medical intervention (Figure 1.1).

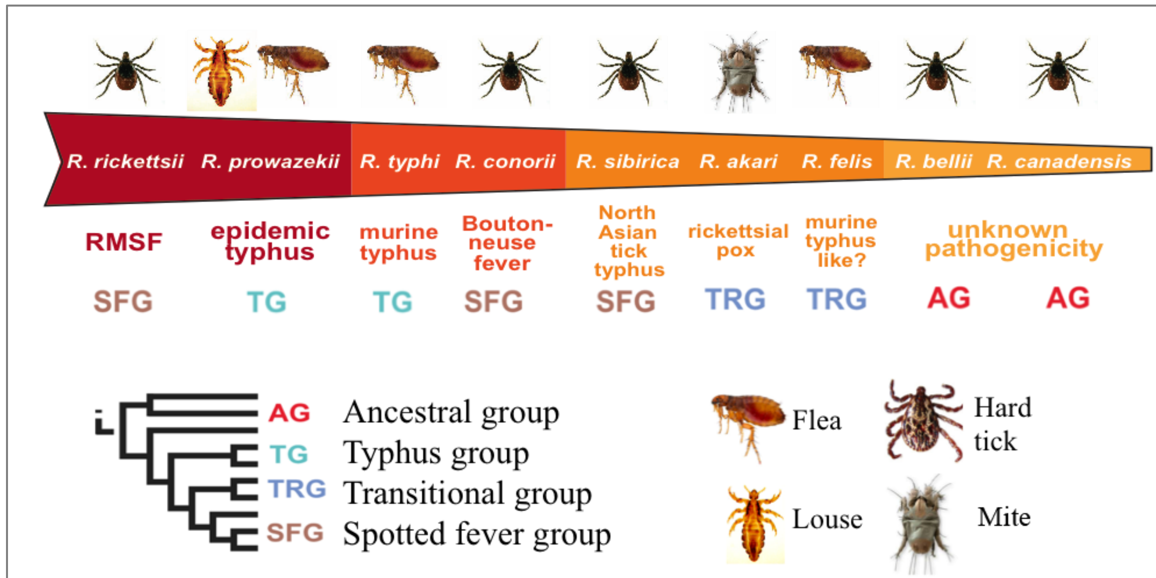

Figure 1.1 Variation in severity of rickettsial disease.
The central graphic represents decreasing virulence, from left to right, of selected rickettsial pathogens. For each pathogenic species, their primary vector is depicted above, with named disease as well as phylogenetic grouping written below. The examples of Rocky Mountain spotted fever (RMSF) and epidemic typhus are described in the text.

Among rickettsial diseases known to humankind, Rocky Mountain Spotted Fever is the most severe. Caused by *R. rickettsii*, RMSF is lethal in 20-30% of cases in the absence of timely diagnosis and antibiotic treatment (9). The bite of *Dermacentor* or *Rhipicephalus* ticks transmits *R. rickettsii* to a human where illness is characterized by fever, headache, myalgia, and macular rash beginning at the wrists and ankles, spreading inward. RMSF and other spotted fevers are often associated with an inoculation eschar at the site of tick attachment. Interestingly, emerging research has indicated that tick salivary components, including immune evasive and anti-coagulant proteins, may facilitate rickettsial survival during the acute phase of rickettsial infection (10, 11). Incidences of Spotted Fever Rickettsiosis reported to the CDC, which includes RMSF

infection, has risen from two cases per million people in the year 2000 to over 11 cases per million in 2014 (12). In addition to the United States of America, RMSF is also endemic in regions of Central and South America, where outbreaks have elicited a state of emergency over public health concerns (13).

The second most severe rickettsiosis is epidemic typhus, caused by *R. prowazekii*. First discovered in the human body louse (*Pediculus humanus humanus*) in 1909 by Dr. Charles Nicolle (14), outbreaks of epidemic typhus have plagued armies, prisons, and impoverished populations where the louse vector thrives (15). With upwards of 60% mortality rate if untreated, some estimates have attributed more than 3 million human deaths to *R. prowazekii* infection in the wake of World War I (16). Owing to its rapid spread through body lice among human populations, epidemic typhus remains an important threat to human health, mainly in communities of poor socioeconomic circumstance, including refugee camps (17). Transmission of *R. prowazekii* does not occur directly through the bite of lice, but by contamination of the bite wound with the feces or crushed body of infected lice. Illness is characterized by flu-like symptoms and a macular rash that begins on the torso before spreading to cover the whole body. Interestingly, *R. prowazekii* is the only rickettsial species known to persist in convalescent patients as a latent infection and manifest years later, a recrudescence known as Brill-Zinsser disease (18).

Once inoculated into the dermis of the skin, pathogenic rickettsiae target vascular endothelial cells preferentially during infection of mammalian hosts (15). Mononuclear phagocytes are also a target, and their colonization helps to transport rickettsiae around the body as they migrate through the lymphatic system and into nearby draining lymph

nodes, from which rickettsiae can enter the blood stream and spread systemically (19, 20). Regardless of species or vector, the outcome of fatal rickettsiosis is the same. Common disease manifestations include hemorrhagic rash, encephalitis, organ failure, pulmonary edema, and severe hypotension due to widespread vascular injury and disseminated inflammation.

At the cellular level during rickettsial disease, endothelial cells are activated at foci of infection, which initiates expression of proadhesive, prothrombotic, and proinflammatory genes (21). This activated state is driven by engagement of NF-κB, STAT1/3, and p38 MAPK/ATF2 transcription factors, which leads to inflammatory cytokine production and infiltration of immune cells to infected tissues (22–26). In a mouse model of rickettsiosis, production of gamma-interferon, the chemokines CXCL9/CXCL10, and recruitment of $CD8^+$ T-cells are crucial for bacterial clearance (27). How these immune responses are initiated by rickettsial infection is poorly understood. Recent work investigated pattern recognition receptor (PRR) engagement during rickettsial infection and found that control of rickettsial burden and disease resolution is driven, in part, by Toll-like receptor (TLR) activation. Both Toll-like receptor 2 (TLR2) and Toll-like receptor 4/lymphocyte antigen 96 (TLR4/MD2) are activated by rickettsiae in reporter cell lines (28), and TLR4/MD2 contributes to bacterial clearance *in vivo* through the MyD88 mediated-signaling (29, 30). However, the molecular mechanism of TLR engagement and activation during infection are unknown.

1.3 Molecular mechanisms of pathogenesis

In order to successfully colonize a host, rickettsiae must rapidly escape immunological defenses and invade their target cells. This process begins with the early evasion of host innate immunity followed by the adhesion and induced phagocytosis. In the latter step, metabolically active rickettsiae cause cytoskeletal rearrangements within the host, culminating in the engulfment of the bacteria into a membrane enclosed phagosome. This *Rickettsia*-containing phagosome is rapidly disrupted, minutes after entry, in the final step of phagosomal escape as rickettsiae enter the replicative niche of the cytosolic compartment (Figure 1.2). The molecular mechanisms of these steps are detailed below.

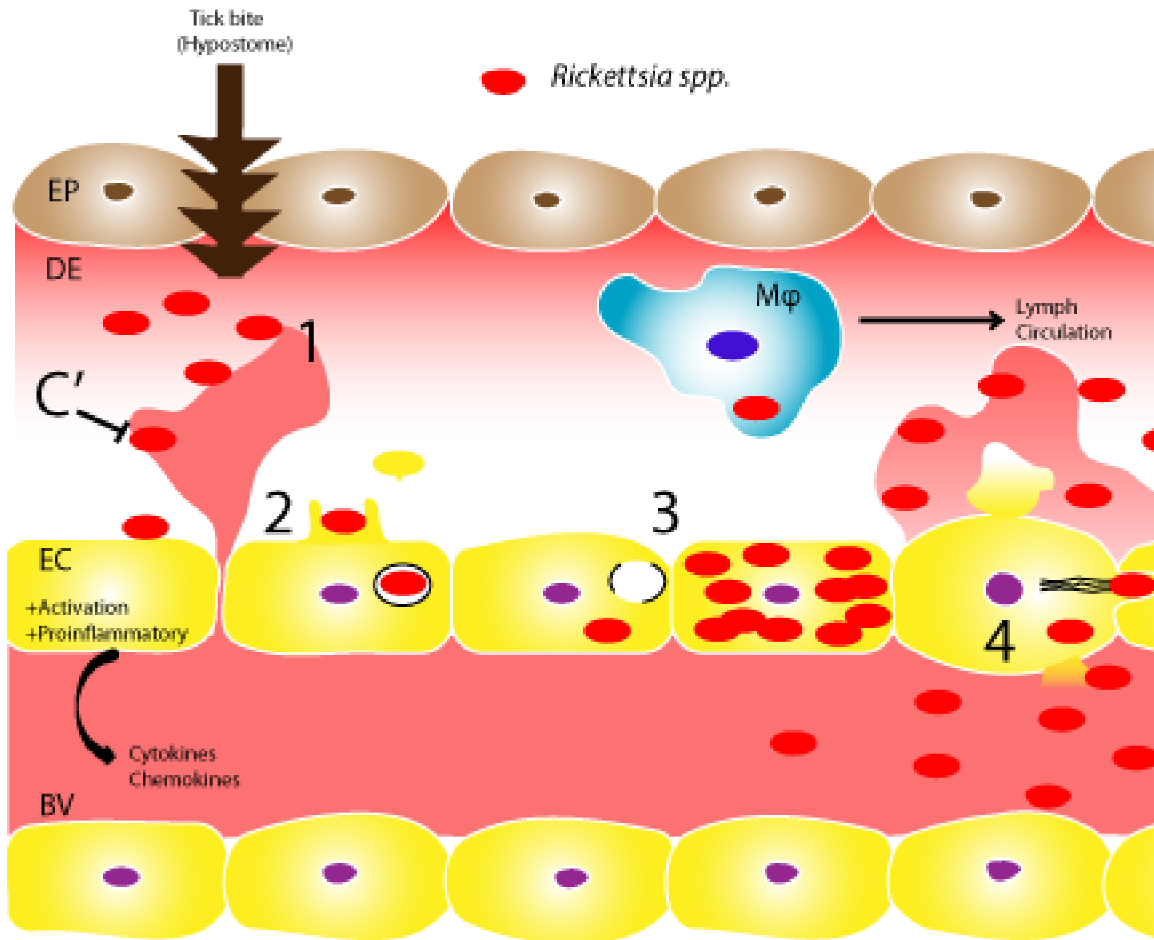

Figure 1.2 *Rickettsia* life cycle.
An inoculum of *Rickettsia* enters the sub-dermal tissue of a mammalian host in the saliva of an infected arthropod; e.g. ticks. The biting tick pictured here creates a small pool of blood around the hypostome, into which host defense-inhibiting molecules and rickettsiae are injected (31). Upon inoculation, bacterial colonization requires (1) evasion of host defenses, such as complement, in the tick feeding pool, (2) attachment and invasion of vascular endothelial cells and resident/infiltrating phagocytes, (3) escape from the phagosome into the replicative niche of the cytosolic compartment, and (4) escape through cell rupture or cell-cell spread via actin based motility (32). During the colonization process, endothelial cells surrounding the infection foci are activated and express inflammatory cytokines as well as chemoattractant chemokines. Infiltrating or resident phagocytes also become infected and can shuttle rickettsiae to other tissues through lymphatic circulation into draining lymph nodes. EP: Epidermis, DE: Dermis, C': Complement proteins, EC: Endothelial cell, BV: Blood vessel, Mφ: Macrophage

1.3.1 Evasion

Exposure to host serum prior to invasion of target cells subjects rickettsiae to the antibacterial activity of the complement system. *R. conorii* resists complement-mediated killing by recruitment of host proteins which disrupt deposition of C3b on the outer membrane (OM) and/or prevention of Microbial Attack Complex-mediated pore formation (Complement reviewed in 8). These resistance mechanisms are carried out by the secreted effectors Adr1and Adr2, as well as Sca5 (OmpB), which recruit host proteins Vitronectin and Factor H, respectively (34–36). Though yet proven, a similar mechanism could protect other rickettsial pathogens from serum-mediated killing during the extracellular phase of infection given that Adr1, Adr2, and Sca5 are conserved in rickettsial pathogens (37).

1.3.2 Adhesion and Induced Phagocytosis

Concurrent to complement defense, rickettsiae initiate entry into host cells via adherence to the extracellular face of target cells and inducing phagocytosis. This is accomplished through adhesion molecules secreted to the rickettsial surface. A recent bioinformatics analysis has revealed 17 Surface Cell Antigens (Scas) encoding autotransporter domains characteristic of Type V secretion system effectors (37). To date, four of these Scas: Sca0 (OmpA), Sca1, Sca2, and Sca5 (OmpB), are implicated in mediating adhesion and/or invasion of several *Rickettsia* species (38–41). In addition, the rickettsial surface protein Adr2 is predicted to act as an adhesins based on preliminary

work using blocking mABs in a reporter system (42). Interestingly, Sca5 and Adr2 play a role in both cellular adhesion as well as complement resistance, highlighting effector multifunctionality as a counterbalance to of reductive genome evolution. After adherence, metabolically active rickettsiae induce their own uptake, causing cytoskeletal rearrangements at entry foci. In *R. conorii*, Ku70/integrin mediated tyrosine kinase activation has been implicated in triggering the Arp2/3 complex, leading to localized actin polymerization. Additionally, the Type IV secretion system effector, RalF, co-ops phosphoinositide metabolism, transiently enriching $PI(4,5)P_2$ in the inner leaflet of the cytoplasmic membrane, a process known to be involved in endocytosis and actin remodeling. RalF is absent in SFG rickettsiae, however similar mechanisms of hijacking lipid metabolism are likely employed by these species. Two pathways of attachment and induction of phagocytosis are proposed by Rennoll-Bankert et al. in (43) based on the available body of literature addressing rickettsial entry. These are depicted in Figure 1.3 and represent general pathways for TG and SFG host cell invasion.

1.3.3 Phagosomal escape, replication, and spread

Internalization of rickettsiae into host cells is very rapid, occurring within minutes after initial contact with the target cell surface. Once internalized into an early phagocytic vesicle, rickettsiae employ membranolytic enzymes to disrupt the phagosomal membrane and enter the host cytosol. Though difficult to observe directly, to date four genes have been characterized to have a role in phagosomal escape. Hemolysin C and phospholipase D, encoded by *tly*C and *pld*A genes, respectively, have been characterized in a

Salmonella enterica heterologous reporter system (44, 45). These genes conferred to *S. enterica* the ability to escape the vacuole, indicating a role in cytosolic entry (46), though knockout of *pld*A in *R. prowazekii* had no observed effect on phagosomal escape in cell culture (47). Secondly, two patatin-like proteins (Pat1 and Pat2), which possess phospholipase A_2 activity (48), have been shown to support intracellular growth and facilitate phagosomal escape (49).

Escape from the phagosome leaves rickettsiae free in the nutrient rich cytosol where they replicate to high numbers before escaping the host cell. Some *Rickettsia* species (not TG rickettsiae) can spread between cells by actin based motility (reviewed in (50)), but all rickettsial species ultimately destroy their host cell *in vitro*, which is a likely *in vivo* mechanism of re-entery into the host blood stream, restarting the infection cycle. Adaptation to intracellular parasitism has streamlined the genome (~1.0-1.5 Mb) through reductive evolution, as metabolic independence has given way to piracy of a myriad of host-derived nutrients in the host cytosol. It is estimated that rickettsiae require uptake of 52 metabolites from their host in order to survive, making *Rickettsia* species the quintessential bacterial parasites of eukaryotic cells (51).

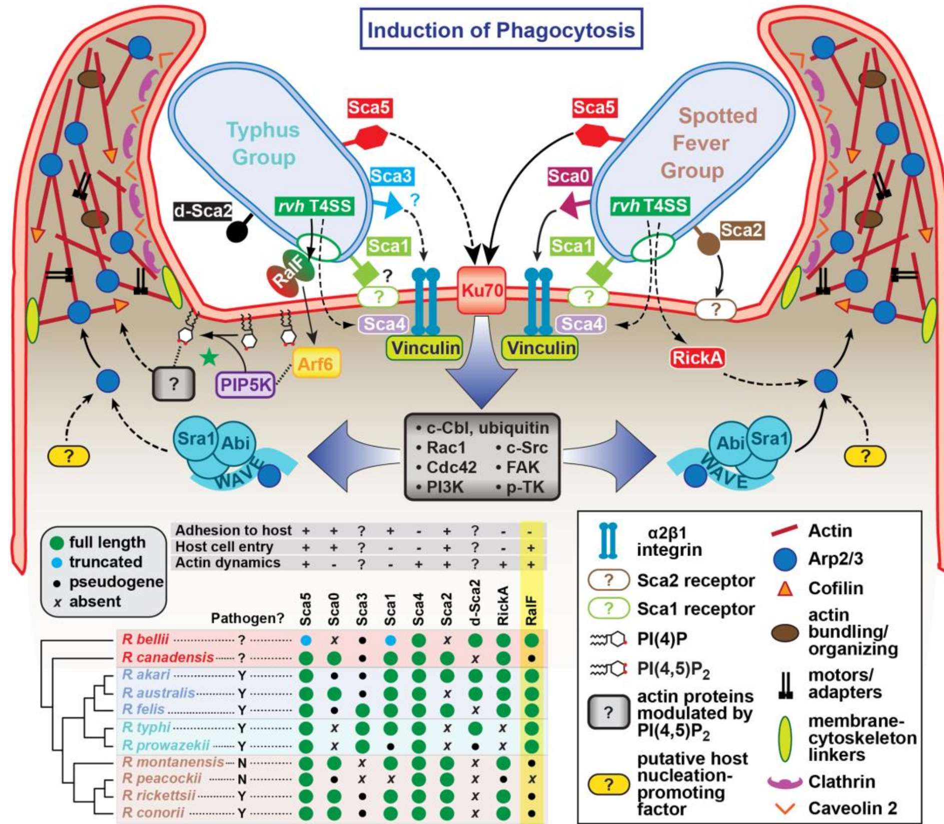

Figure 1.3 Model for the variable pathways utilized by divergent *Rickettsia* species for host cell entry. General pathways for Typhus Group (TG, left) and Spotted Fever Group (SFG, right) rickettsiae species are inferred primarily from previous work on SFG rickettsiae species *R. conorii* and *R. parkeri* or data Rennoll-Bankert et al. (43, 52) (*R. typhi*). At center, a conserved proximal hub of the pathway commences with Sca5 binding to host receptor Ku70, which triggers a host-signaling cascade (gray box) involving c-Cbl-mediated ubiquitination of Ku70, Rho-family GTPases Cdc42 and Rac1, phosphoinositide 3-kinase (PI3K) activity, and activation of tyrosine kinases (e.g., c-Src, FAK and p-TK) and their phosphorylated targets. The divergent distal arms of this pathway involve recruitment of factors for activating the actin nucleating complex (Arp2/3), which leads to host actin polymerization, extensive membrane ruffling and filopodia formation, and bacterial internalization in a clathrin and calveolin dependent process. For SFG rickettsiae, the WAVE complex recruits Arp2/3, with its activation via an unknown nucleation-promoting factor (either host or bacterial; e.g., RickA). While these processes remain to be characterized for TG rickettsiae, our work suggests that secreted RalF recruits the GTPase Arf6, precipitating an accumulation of PI(4,5)P2 that modulates the activities of a range of actin-associated host proteins (green star). Additional bacterial proteins, some of which are known to facilitate host cell entry, have white lettering with colored boxed backgrounds. Known pathways for protein secretion and host cell receptor-binding are shown with solid black lines; all other modeled pathways (shown with dashed lines) are either inferred by homology (e.g., Sca1 of TG rickettsiae as an adhesin based on characterization for Sca1 of *R. conorii*) or estimated based on *in silico* analyses (e.g., Sca3 of TG rickettsiae as a putative analog to the α2β1 integrin-binding Sca0 of *R. conorii*). A phylogenomics analysis across select *Rickettsia* species (bottom, left) illustrates the genomic variation underlying all of the bacterial components of the models. Adapted from our recent report on the Rickettsia secretome (37). Red, ancestral group (AG); blue, transitional group (TRG); aquamarine, TG; brown, SFG. Reprinted from Ref (43) with permission by PLOS. © 2015 Rennoll-Bankert et al. licensed under CC BY 4.0

1.4 Outer membrane and Lipopolysaccharide

Molecular interactions at the host-pathogen interface are required for invasion of target cells. At the nexus of host-pathogen interaction, the rickettsial OM is the foundation on which secreted proteins mediate the above-described steps of evasion, adhesion, and invasion of target cells. Similar to other Gram-negative bacteria, the *Rickettsia* OM is an asymmetric lipid bilayer whose outer-leaflet is predominately composed of lipopolysaccharide (LPS) (53).

LPS plays a critical role in the barrier function of the OM (54) and is composed of three domains: O-antigen, core (separated into outer and inner core), and lipid A (Figure 1.4). The O-antigen is a large glycan polymer of repeating oligosaccharides (2-6 units) containing several different carbohydrates (55). Though highly immunogenic, variation in carbohydrate composition, as well as non-carbohydrate modifications, make the O-antigen heterogeneous in composition between species, or even within strains of a single species of bacteria. The core domain is a non-repeating oligosaccharide typically built upon a backbone disaccharide of 3-Deoxy-D-manno-oct-2-ulosonic acid, also known as KDO. The outer core is commonly composed of hexose residues and links the O-antigen to the inner core, which attaches to lipid A directly through α-ketosidic linkage between the terminal KDO

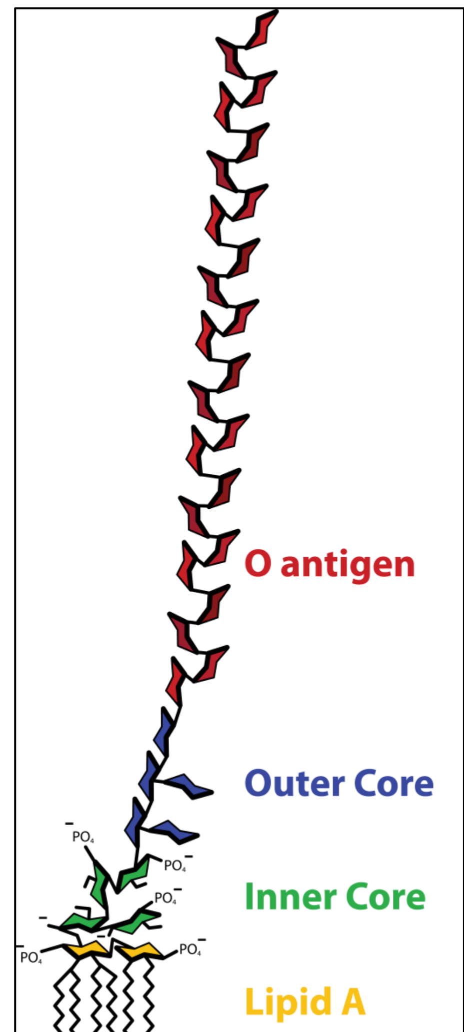

Figure 1.4 General structure of LPS
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residue and the reducing glucosamine of lipid A residues (Figure 1.5, GlnII) (53). LPS structure and modifications are reviewed thoroughly Trent MS *et al.* (56).

Lipid A the most conserved component of LPS and is the hydrophobic moiety which anchors the molecule into the outer leaflet of the OM. With few exceptions, lipid A and KDO sugars are required for growth of Gram-negative bacteria (57, 58). Most bacteria synthesize a lipid A molecule that is a bisphosphorylated glucosamine disaccharide backbone, decorated with fatty acids resembling the canonical lipid A synthesized by *E. coli* depicted in Figure 1.5. Though the most conserved component of LPS, divergent lipid A structures have evolved in Gram-negative bacteria. Modification systems and biosynthetic diversity are known to drive changes in lipid A architecture as bacteria acclimate to their environment (59). As an adaptation to a particular ecological niche, or as a pathogen's defense against a lethal host immune system, variations in lipid A structure can markedly change the OM properties as well as the inflammatory capabilities of the molecule (60, 61).

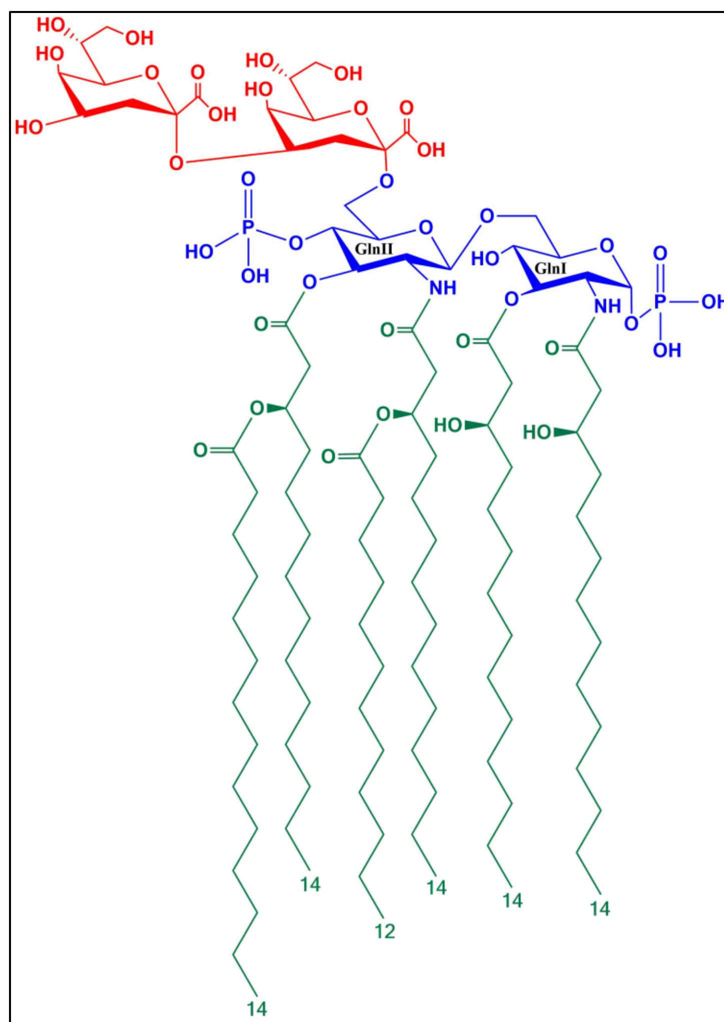


Figure 1.5 Typical structure of Lipid A.
KDO sugars of the inner core are in red, with the Lipid A glucosamine backbone and acetly chains in blue and green, respectively.

This lipid A moiety is detected by the TLR4/MD2 receptor complex on the surface of mammalian immune cells, and within immune cells by caspases of the noncanonical inflammasome (Casp-11 in mice, Casp-4/5 in humans) (62, 63). Minute quantities of lipid A are capable of eliciting an inflammatory response, driving immune cell activation and expression of bactericidal molecules (59). For *Rickettsia* species, TLR4/MD2 is activated during infection as described above. Additionally, Caspase-11

activation may play a role in a mouse model of *R. australis* infection (64), both indicating an endotoxic component to rickettsial infection. Lipid A is the classical endotoxic PAMP of Gram-negative bacteria. **However, next to nothing is known about the biology of lipid A in *Rickettsia* species**. The work carried out in this dissertation addresses critical gaps in our knowledge of rickettsial OM biology by;

i) Describing the variation in lipid A structure between *Rickettsia* species. Given the differences in host colonization, as well as the drastic differences in virulence between *Rickettsia* species, I hypothesize that rickettsiae have evolved dissimilar lipid A architecture as a consequence of species divergence.

ii) Characterizing two proteins from *R. typhi* and *R. rickettsii* that are members of a newly described family of lipid A acyltransferases predicted to be present in all *Rickettsia* species. I hypothesize that *Rickettsia* expresses the late acyltransferase LpxJ in order to complete lipid A biosynthesis by the addition of C14/C16 secondary fatty acids.

I believe that understanding the membrane dynamics that facilitate molecular interactions at the host-pathogen interface, which are required for adhesion and invasion of mammalian cells, are imperative to the field of rickettsiology. This work reveals novel insight into rickettsial pathogenesis and contributes greatly to our understanding of rickettsial physiology.

CHAPTER 2: DIVERSITY OF LIPID A WITHIN RICKETTSIA

2.1 Introduction: Overview of lipid A

LPS, an amphipathic molecule composed of three domains (described above), comprises the majority of the outer leaflet of the OM of Gram-negative bacteria. The LPS of *Rickettsia* species is of the smooth type (O-antigen-containing) and is highly immunogenic, with SFG rickettsiae LPS having strong cross-reactivity to antibodies raised against *Proteus vugaris* (Enterobacteriacea) (65). Several studies have investigated the carbohydrate composition of the *Rickettsia* O-antigen and core domain, but little is known about the lipid A moiety of rickettsial LPS (65–69). As described above, lipid A is hydrophobic and anchors LPS into the OM. It is also the most conserved portion of LPS and often a potent activator of the mammalian immune system through ligation of TLR4/MD2 (70, 71), as well as activation of the noncanonical inflammasome (72). But not all lipid A molecules are equal in their ability to activate these PRRs (73). Most heretofore analyzed Gram-negative bacteria synthesize *E. coli*-like lipid A, which is the classical PAMP for these receptors and one of the most inflammatory molecules known to humankind (59). However, lipid A structural divergence can reduce or abolish TLR4/MD2 signaling. Indeed, several bacterial pathogens utilize lipid A modification as a mechanism of immune evasion when infecting a mammalian host (74–77). Further, lipid A structure is known to control membrane charge and permeability, therefore contributing greatly to resistance of membrane-directed anti-microbial peptides, as well as antibiotic therapies (78–80). Given the apparent importance of lipid A in the

development of protective immunity, as well as therapeutic interventions during a bacterial infection, it is critically important to understand the form and function of lipid A of Gram-negative pathogens.

Prior studies have shown that *R. akari* and *R. conorii* activate TLR4/MD2 during host cell infection, leading to suppositions that lipid A is the endotoxic component of rickettsial LPS (28, 81). However, only a single description of *Rickettsia* lipid A structure has been published (82). This work, which analyzed an unreported strain of *R. typhi*, identified a lipid A molecule that is hexa-acylated and bisphosphorylated, with fatty acids ranging from C14 to C18 in length (Figure 2.1). Given the divergence in lipid A structure reported within other genera of Gram-negative bacteria (83), including other intracellular pathogens (84), it is important to understand the variation in lipid A between *Rickettsia* spp. as a possible correlate of disease severity. In this chapter, I report the lipid A structure of four diverse species of *Rickettsia,* including a previously unreported lipid A structure found in *R. rickettsii* Str. Shiela Smith, the most virulent rickettsial pathogen. Because lipid A architecture is fundamental to OM integrity, this foundational work contributes greatly to our understanding of rickettsial physiology.

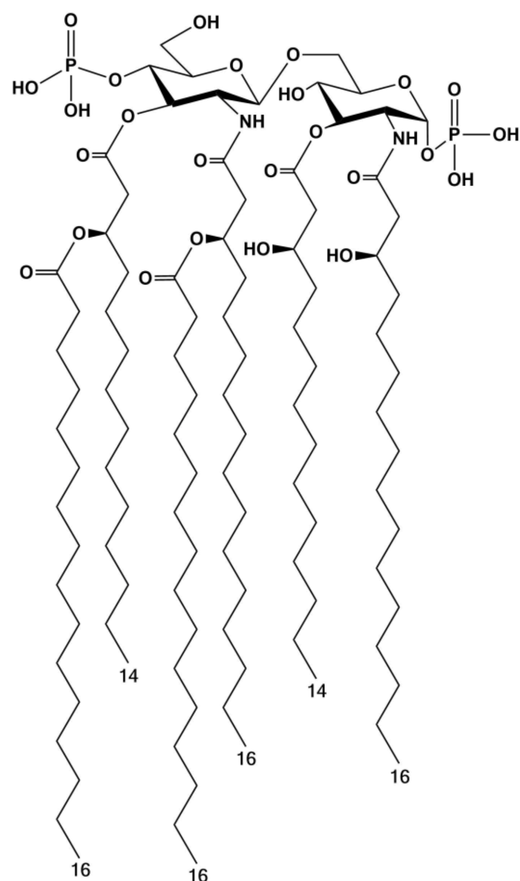


Figure 2.1 Lipid A of *R. typhi* redrawn from (82)

2.2 Results

In order to investigate the diversity of lipid A between rickettsiae, we cultured four species that are divergent both ecologically and pathologically (Figure 1.1). *R. typhi* str. Wilmington*, R. montanensis, R. akari* str. Hartford*,* and *R. rickettsii* str. Sheila Smith were cultured in monolayers of Vero76 host cells. Lipid A was extracted from each culture and subjected to MALDI-TOF analysis. Masses of lipid A ions taken from these species were compared to the previously reported structure of *R. typhi* lipid A (Figure 2.1).

R. typhi str. Wilmington, *R. montanensis,* and *R. akari* str. Hartford have major lipid A ions at m/z 1936-37 (Figure 2.2 A-C). These ions likely represent the

bisphosphorylated hexa-acyl structure depicted in Figure 2.1 that has been previously reported (82), indicating a conserved lipid A architecture between these species. However, there is considerable variance between *R. typhi, R. montanensis,* and *R. akari* in the number and intensity of minor lipid A ions. Minor lipid A peaks around m/z 1963, 1910, and 1881 represent heterogeneity in fatty acid incorporation into lipid A molecules; a phenomenon known to occur in other Gram-negative bacteria (85–88). The reason for variance in minor lipid A species between these rickettsiae is unknown but warrants further investigation.

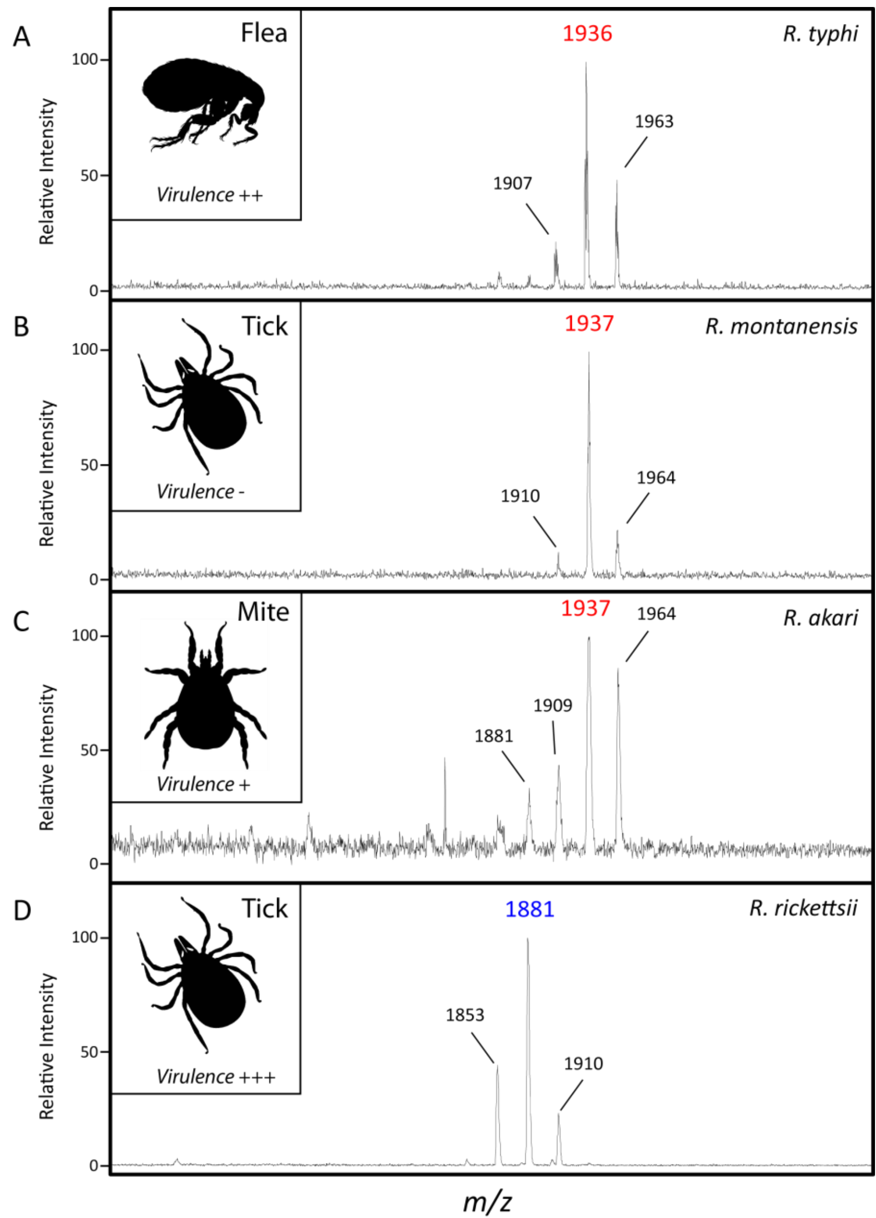


Figure 2.2 MALDI-TOF analysis of lipid A.
(A) *R. typhi* Str. Wilmington, (B) *R. montanensis*, (C) *R. akari* Str. Hartford, and (D) *R rickettsii* str. Sheila Smith. Insets identify the vector host and relative virulence to humans. Peak labels are masses of singly charged ions. Major peaks are indicated by colored labels.

Interestingly, the most pathogenic species of *Rickettsia* tested in this work, *R. rickettsii*, produces a major lipid A molecule that is less massive than the other three species (major ion m/z 1881 Figure 2.2 D). This 56 Dalton reduction constitutes a difference in fatty acid length corresponding to a loss of 4 carbons from one or more fatty acid chains. In order to identify divergence in fatty acid incorporation, we subjected lipid A of *R. rickettsii* (Figure 2.3 A) to sequential fatty acid release prior to MALDI-TOF analysis of daughter molecules (see Methods; Figure 2.6). Ester-linked fatty acids that are directly attached to the di-glucosamine backbone are released more readily than secondary ester-linked fatty acids. Using mild alkali treatment with ammonium hydroxide (89), we first liberated primary ester-linked fatty acids (Figure 2.6; Red acyl chains) yielding a predominate ion at m/z 1190, which is a tri-acyl daughter molecule (Figure 2.3 B). Next, a second alkaline reaction with methylamine removed the last remaining secondary ester-linked fatty acid (Figure 2.6; Blue acyl chain) attached to the hydroxypalmitate at the 2’ position. A loss of 182 Daltons of mass represents the liberation of a laurate (C12) from this position, yielding the di-acyl daughter ion at m/z 1008 (Figure 2.3 C).

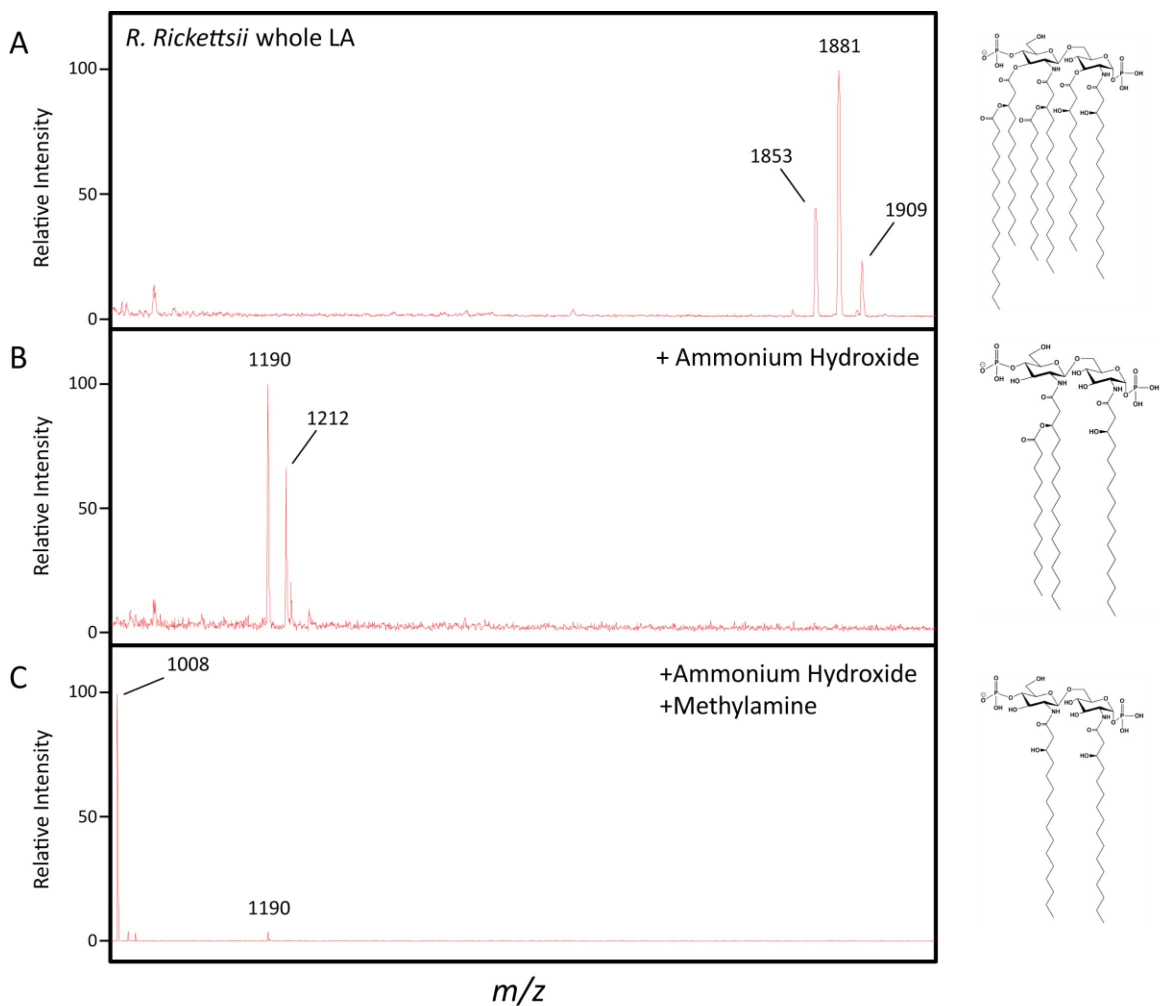


Figure 2.3 Fatty acid analysis of lipid A from *R. rickettsii* str Sheila Smith.
(A) whole lipid A, (B) lipid A derivatives after treatment with ammonium hydroxide, (C) lipid A derivatives after treatment with ammonium hydroxide then methylamine. Molecules on the right depict the major lipid A ion in each spectrum. Minor peaks likely represent heterogeneity in fatty acid incorporation

2.3 Discussion and Future Directions

In this chapter we have characterized lipid A from four rickettsiae. *R. montanensis*, *R. akari* str. Hartford, and *R. typhi* str. Wilmington have the same lipid A structure, independent of reservoir host or pathogenicity. Additionally, we have identified a novel lipid A structure form the highly virulent *R. rickettsii* str. Sheila Smith. This lipid A molecule is hexa-acylated and bisphosphorylated, similar to other characterized rickettsiae, however, the secondary fatty acid coupled to the hydroxypalmitate (C16-OH)

at the 2’ position is a laurate (C12) compared to palmitate/sterate (C16/C18) as previously described for *R. typhi* (82) (Figure 2.4). The mechanism of differential fatty acid usage between these species is unknown. The impact of this difference on OM dynamics as well as the inflammatory potential of the molecule are currently unknown but are the focus of my ongoing research.

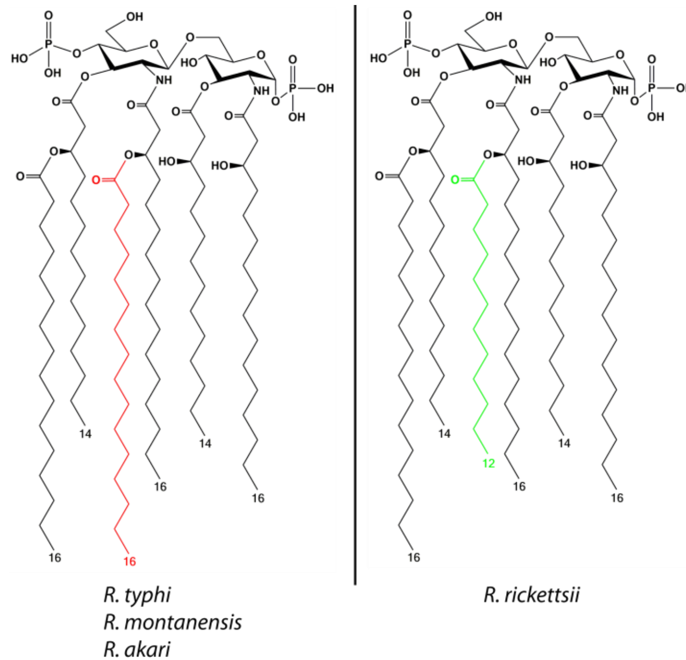
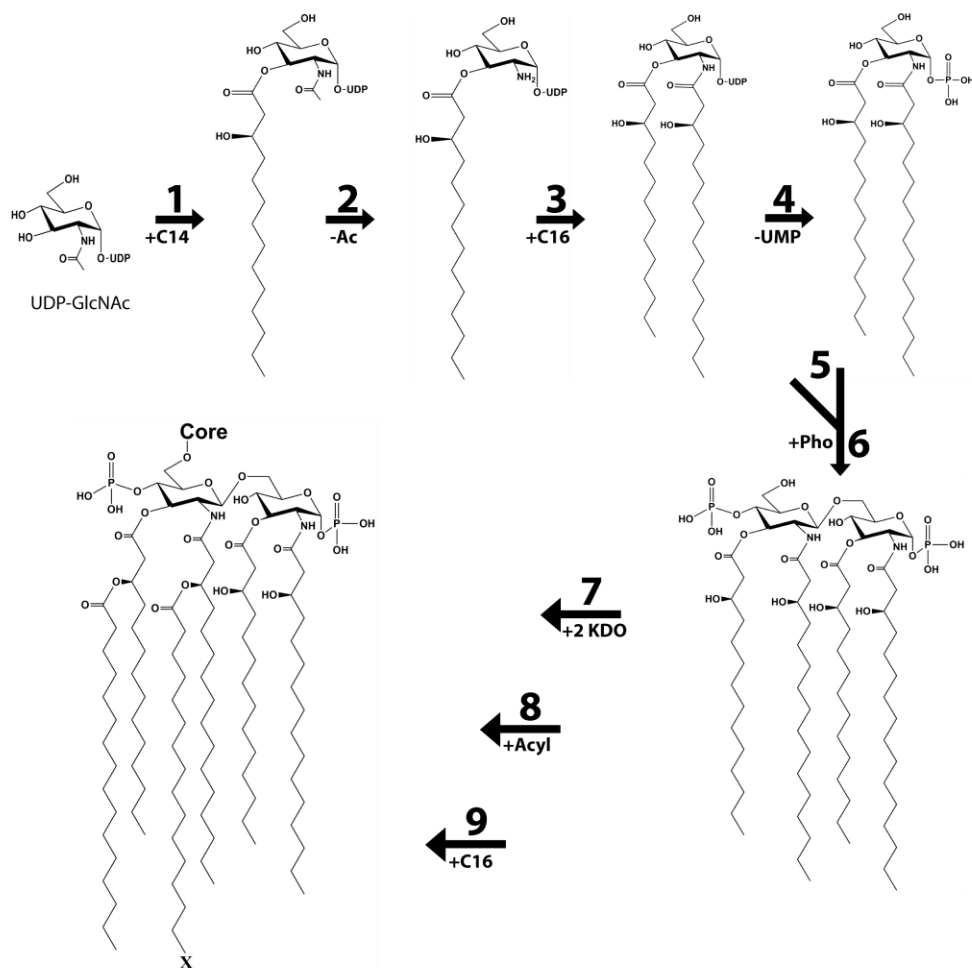


Figure 2.4 Proposed lipid A structures for *R. typhi* str. Wilmington, *R. montanensis*, *R. akari* str. Hartford, and *R. rickettsii* str. Sheila Smith

The characterization of rickettsial lipid A continues to be crucial in order to provide further insight into the molecular mechanisms of host-pathogen interactions, as well as adaptation of the OM to an obligate intracellular lifecycle. Further, given the apparent endotoxicity of rickettsial infection, it is important to understand the inflammatory contribution of lipid A as a potential correlate of disease severity (28, 30). Accordingly, a comprehensive understanding of the molecular pathology of infection is indispensible for development of productive therapeutic and prophylactic interventions that mitigate morbidity and mortality of rickettsial infection.



		R. typhi		*R. montanensis*		*R. akari*		*R. rickettsii*	
Step	Gene	Locus tag	*	Locus tag	C/I	Locus tag	C/I	Locus tag	C/I
1	*lpx*A	RT0006	*	MCI_04185	100/91	A1C_00030	89/88	A1G_00035	100/91
2	*lpx*C	RT0246	*	MCI_05905	99/92	A1C_01850	99/90	A1G_01940	99/91
3	*lpx*D	RT0008	*	MCI_04195	99/92	A1C_00050	99/89	A1G_00045	99/88
4	*lpx*I	RT0716	*	MCI_02840	99/85	A1C_05870	99/84	A1G_06155	99/86
5	*lpx*B	RT0311	*	MCI_06400	99/70	A1C_02380	99/78	A1G_02490	99/68
6	*lpx*K	RT0705	*	MCI_02755	99/84	A1C_05575	99/83	A1G_06070	99/84
7	*kdt*A (*waa*A)	RT0048	*	MCI_04790	99/80	A1C_00640	99/75	A1G_00700	99/80
8	*lpx*L	RT0704	*	MCI_02750	99/91	A1C_05570	99/86	A1G_06065	99/90
9	*lpx*J	RT0047	*	MCI_04795	99/88	A1C_00645	99/85	A1G_00705	99/88

Figure 2.5 Schematic pathway of lipid A biosynthesis.
Each step of the Raetz pathway of lipid A biosynthesis is carried out by a single enzyme (Numbers 1-9). The labeled arrows represent addition of lipid A constituents or removal of metabolic byproducts. The fatty acid labeled with an X in the final product varies in length between *R. rickettsii* and other species of *Rickettsia* as described in the text, but is modeled here as a C16. The table identifies genes of the Raetz pathway for four species of *Rickettsia* whose lipid A were characterized in this study. BLASTP analysis using *R. typhi* primary protein sequences as the query gave homology scores reported under each species (C=query coverage; I=Identity%). C#=Fatty acid of # carbons; Ac=Acetate; UMP=Uridine Monophosphate; Pho=Phosphorylation; KDO=3-Deoxy-D-manno-oct-2-ulosonic acid; Acyl=Acylation.

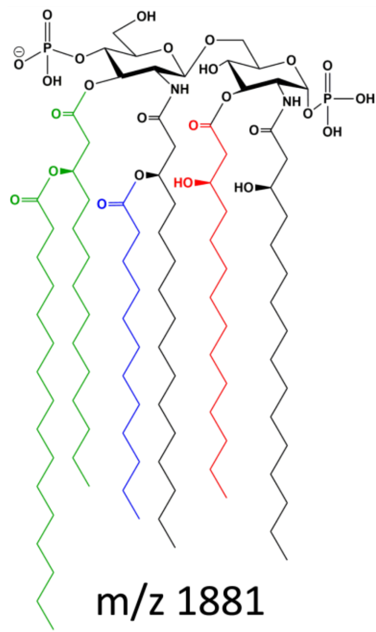
2.4 Methods

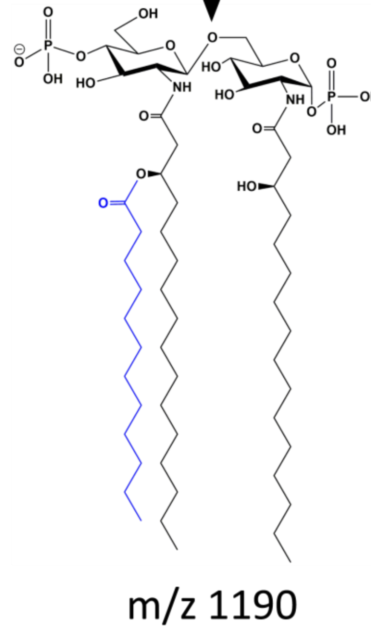
Bacterial strains and cell culture. Vero76 cells (African green monkey kidney, ATCC: CRL-1587) were maintained in Dulbeco's modification of Eagle's medium (DMEM with 4.5 gram/liter glucose and 480 L-glutamine) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C with 5% CO_2. *R. typhi* str. Wilmington (ATCC: VR-144), *R. akari* str. Hartford, *R. montanensis*, and *R. rickettsii* str. Sheila Smith were propagated in Vero76 cells grown in DMEM supplemented with 5% FBS at 34°C with 5% CO_2. Rickettsiae were propagated for 48-72 hours until confluent before harvesting. Rickettsial cultures were partially purified by mild sonication (3 x 10s pulses; power output 6) followed by 5.0 µm filtration and collected by gentle centrifugation (2,000 x g; 15 minutes). Rickettsial pellets were washed once in ultrapure water prior to lipid A extraction.

Lipid A micro-extraction. Micro-extraction of lipid A from *Rickettsia* spp. cultures was performed as previously described (90, 91). Briefly, pellets from 1-4 confluent T75cm^2 culture flasks maintained as described above, were extracted in 400 µL of a solution containing five parts of isobutyric acid: three parts of 1M ammonium hydroxide and heated at 100°C for 1 h followed by a 15-min incubation on ice and centrifugation at 2000 × g for 15 min. The supernatant partitions into two layers, with contaminating cardiolipin remaining exclusively in the top layer and lipid A partitioning to the bottom layer. The bottom layer was collected and mixed in equal parts with water and then frozen and lyophilized. Contaminants were washed from the dried material by two rounds of methanol washes: 1 ml of methanol, sonicating, and pelleting at 10,000 × g for 5 min.

The final product was reconstituted in 2:1:0.25 Chloroform:Methanol:Water (50 µL) along with 4–8 grains of Dowex ion exchange resin (Fisher Scientific, Pittsburgh, PA, USA), incubated at room temperature with vortexing for at least 5 min. Solubilized lipid A molecules (1-2 µL) were spotted onto a stainless steel target plate along with 1 µL of Norharmane matrix (10mg/ml in 2:1 C:M) for MALDI analysis on a Bruker MicroFlex matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry instrument in negative-ion mode calibrated with Agilent Tuning Mix (Santa Clara, CA, USA. G2421A) and data were processed in flexAnalysis (Bruker Daltonics). All microextraction chemicals were obtained from Sigma-Aldrich unless otherwise noted.

Sequential fatty acid release by alkaline treatment. To liberate primary ester-linked fatty acids, lipid A samples prepared as above were suspended in 100 µl of 28% ammonium hydroxide (Sigma; 221228) and incubated at 50°C for 5 hours with occasional vortexing. To liberate secondary fatty acids, lipid A or previously treated derivatives, were suspended in 40% methylamine (Sigma 426466) and incubated at 50°C for 3 hours. 100 µl of ultrapure water was added before samples were frozen and lyophilized. Final products were reconstituted in chloroform:methanol:water and analyzed by MALDI-TOF as described for whole lipid A above (Figure 2.6).





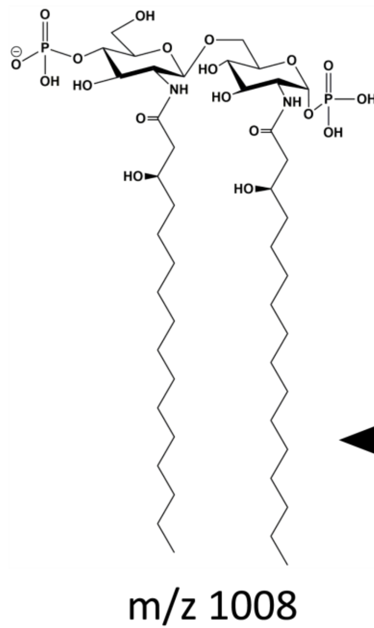


Figure 2.6 Schematic representation of analytical methods used to determine fatty acid composition. Lipid A from *R. rickettsii* str. Sheila Smith was subjected to sequential release of fatty acids. m/z=mass-to-charge ratio of lipid A derivative ions identified during MALDI-TOF analysis described in the text.

CHAPTER 3: RICKETTSIA LIPID A BIOSYNTHESIS UTILIZES THE LATE ACYLTRANSFERASE LPXJ FOR SECONDARY FATTY ACID ADDITION[1]

3.1 Abstract

Members of the *Rickettsia* genus are obligate intracellular, Gram-negative coccobacilli that infect mammalian and arthropod hosts. Several rickettsial species are human pathogens and are transmitted by blood-feeding arthropods. As Gram-negative parasites, the outer membrane (OM) sits at the nexus of host-pathogen interaction and is rich in lipopolysaccharide (LPS). The lipid A component of LPS anchors the molecule to the bacterial surface and is an endotoxic agonist of Toll-like receptor 4 (TLR4). Despite the apparent importance of lipid A in maintaining OM integrity, as well as its inflammatory potential during infection, this molecule is poorly characterized in *Rickettsia* pathogens. In this work, we have identified and characterized new members of the recently discovered LpxJ family of lipid A acyltransferase in both *Rickettsia typhi* and *Rickettsia rickettsii*, the etiological agents of murine typhus and Rocky Mountain Spotted Fever, respectively. Our results demonstrate that these enzymes catalyze the addition of a secondary acyl chain (C14/C16) to the 3'-linked primary acyl chain of the lipid A moiety in the final steps of the Raetz pathway of lipid A biosynthesis. Because lipid A architecture is fundamental to bacterial OM integrity, I believe rickettsial LpxJ may be important in maintaining membrane dynamics to facilitate molecular interactions at the

[1] Mark L. Guillotte*, Joseph J. Gillespie, Courtney E. Chandler, M. Sayeedur Rahman, Robert K. Ernst, Abdu F. Azad. *Journal of Bacteriology* (Accepted; 16 July 2018)

host-pathogen interface that are required for adhesion and invasion of mammalian cells. This work reveals novel insights into *Rickettsia* pathogenesis and contributes to our understanding of rickettsial OM structure and physiology.

3.2 Introduction

Species of *Rickettsia* (Rickettsiales: *Alphaproteobacteria*) are Gram-negative obligate intracellular parasites of a vast range of eukaryotes (92). While mechanisms for host cell invasion are variable across rickettsial lineages (43), all rickettsiae lyse the host phagocytic vacuole and reside primarily in the host cytosol (93). Dependent on a plethora of host metabolites, rickettsiae have a diminished metabolic capability relative to free living and facultative intracellular bacteria, as well as vacuolar obligate intracellular species (51). Thus, while rickettsiae vary in their ability to infect vertebrates and cause pathogenesis, all species are metabolic parasites of the eukaryotic cytoplasm.

Remarkably, despite the lack of glycolytic enzymes, rickettsiae synthesize a typical Gram-negative bacterial cell envelope (65, 67, 94). The inner membrane (IM) and outer membrane (OM) are separated by a relatively thin peptidoglycan layer (95), which contains diaminopimelate in the stem peptide (96). The OM is asymmetric, with the inner leaflet composed mainly of glycerophospholipids (97, 98) and the outer leaflet predominantly composed of lipopolysaccharide (LPS) (69, 99, 100). As the interface connecting host to microbe, the rickettsial OM constituents are critically important not only for host cell invasion, but also for mediating intracellular survival. The composition of surface proteins has been identified for several species (101–104) and nearly two dozen effectors are recognized as components of the secretome (37), however the non-

proteinaceous surface components of *Rickettsia*, including LPS are critically understudied.

LPS is highly antigenic in rickettsial infection (105, 106) and is composed of an outer O-antigen polysaccharide linked to a core oligosaccharide, which is anchored in the bacterial outer leaflet by lipid A (73). Lipid A is a pathogen associated molecular pattern (PAMP) known for its ability to trigger an inflammatory response through its interaction with the Toll-like receptor 4/myeloid differentiation factor 2 (TLR4/MD2) complex, as well as activation of the non-canonical inflammasome through cytosolic caspases (Casp-4/5 in humans; Casp-11 in mice) (71, 72, 107). In a mouse model of Rickettsiosis, TLR4/MD2 activation is critical for bacterial clearance (81, 108); however, little is known about the contribution of LPS to the inflammatory nature of *Rickettsia* infection (66, 109). Recent analysis of the lipid A from *R. typhi* revealed structural differences relative to the highly inflammatory lipid A of *E. coli*, though the potential of rickettsial lipid A to act as a TLR4 agonist remains unclear (82) (Figure 3.1).

Our recent phylogenomics study indicated rickettsiae contain a nearly conserved Raetz pathway for the synthesis of lipid A, lacking only the late acyltransferase LpxM (51). LpxM, which catalyzes the 3′-secondary acylation of KDO_2-lauroyl-lipid IV_A (typically transferring myristate) subsequent to 2′-secondary acylation (carried out by LpxL), is absent in many bacteria, some of which alternatively carry a non-orthologous late acyltransferase named LpxJ (previously named DUF374) (110–112). LpxJ enzymes of *Helicobacter pylori*, *Campylobacter jejuni,* and *Wolinella succinogenes* (all *Epsilonproteobacteria* pathogens) catalyze 3′-secondary acylation, but can or must do so prior to 2′-secondary acylation (LpxL) and even 3-deoxy-D-manno-octulosonic acid

(KDO) transfer (carried out by the KDO transferase WaaA). Thus, LpxJ enzymes can be considered more functionally promiscuous than their LpxM counterparts. As LpxJ homologs are present in all *Rickettsia* genomes, with the *R. typhi* enzyme sharing 27% identity to *H. pylori* LpxJ, we reasoned that these enzymes complete the Raetz pathway for rickettsial lipid A biosynthesis and incorporate a C16 fatty acid chain as a 3'-secondary acylation (82).

Herein, we provide enzymatic evidence that *Rickettsia* LpxJ complements *E. coli* LpxM mutants and carries out 3′-secondary acylation of lipid IV_A and lauroyl-lipid IV_A. Additionally, targeted mutagenesis based on comparative analysis of >2800 DUF374 family members with LpxJ homologs reveals residues critical for acylation. In line with prior work (110), our data demonstrates that divergent LpxJ and LpxM active sites both catalyze 3′-secondary acylation for lipid A biosynthesis and that LpxJ is a non-orthologous replacement of LpxM in a vast range of diverse bacteria. As lipid A architecture is fundamental to bacterial OM integrity, our findings indicate that LpxJ is important in maintaining ideal membrane dynamics to facilitate molecular interactions at the host-pathogen interface.

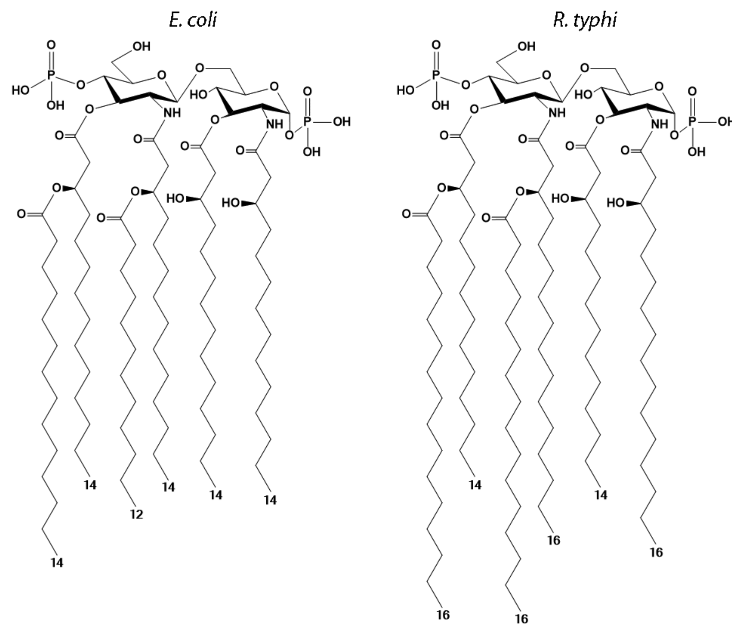


Figure 3.1 Lipid A structures of *Escherichia coli* and *Rickettsia typhi*.

3.3 Results

***Rickettsia* encode a homolog of LpxJ.** Rickettsial comparative genomic analysis has identified a nearly complete Raetz pathway of lipid A biosynthesis (Supplemental Figure 5.1). However, *Rickettsia* species do not encode any enzymes similar to LpxM (also known as MsbB). Since *R. typhi*, (and probably all species of *Rickettsia*) produce hexa-acylated lipid A (Figure 2.5 (82)), we reasoned that a lipid A acyltransferase analogous to LpxM has escaped gene annotation within rickettsial genomes. In their report describing LpxJ, Rubin *et al*. identified a putative LpxJ homolog in *R. rickettsii* (27% similarity at the protein level) (110). We have further identified LpxJ family genes throughout genus *Rickettsia* (Table 3.1) and have selected putative homologs from *R. typhi* (RT0047) (Supplemental Figure 5.2) and *R. rickettsii* (A1G_00705), hereafter $LpxJ^{Rt}$ and $LpxJ^{Rr}$, for molecular characterization.

LpxJ^{Rt} and LpxJ^{Rr} complement an *E. coli* LpxM mutant. In order to investigate the role of LpxJ in *Rickettsia* lipid A biosynthesis, we utilized a heterologous system in which acylation-deficient lipid A mutants of *E. coli* act as a reporter of enzyme function for exogenously expressed acyltransferases. We first expressed LpxJ^{Rt} and LpxJ^{Rr} in an *lpxM* mutant MLK1067 that elaborates predominately penta-acylated lipid A. After inducing expression of rickettsial proteins (Supplemental Figure 5.3), lipid A extractions were prepared and subjected to MALDI-TOF analysis to determine if rickettsial LpxJ can complement the loss of *lpxM* and produce hexa-acyl lipid A. In comparison to untransformed MLK1067, we observed additional lipid A species of increased mass from cells expressing LpxJ^{RT} and LpxJ^{Rr}, but no change from cultures transformed with an empty plasmid vector (Figure 3.2). The ions at *m/z* 1797 and *m/z* 1825 represent the addition of C14 (Δm/z 210) or C16 (Δm/z 238), respectively, to the parental penta-acylated lipid A (m/z 1587). MALDI-TOF results for LpxJ^{Rt} were confirmed using gas chromatography. Fatty acid peaks were identified by comparison to commercially available bacterial acid methyl ester (BAME) standards. The amount of each fatty acid present in lipid A was calculated by comparison to an internal pentadecanoic acid (C15) standard. LpxJ^{Rt} overexpressing-cells show a four-fold increase in the total amount of C14 and C16 (Figure 3.3). Taken together, these data indicate that LpxJ^{Rt} and LpxJ^{Rr} are bona fide acyltransferases in the lipid A pathway.

Table 3.1 Conservation between rickettsial LpxJ homologs.

Species (Strain)	Locus Tag	Homology (I%,P%,G%)	E-value
R. typhi (Wilmington)	RT0047	100,100,0	2e-161
R. prowazekii (Breinl)	H375_5410	98,99,0	8e-159
R. rickettsii (Sheila Smith)	A1G_00705	88,92,0	2e-140
R. felis (LSU)	JS55_00590	89,93,0	1e-142
R. akari (Hartford)	A1C_00645	85,91,0	1e-136
R. belli (RML Mogi)	RBEMOGI_1439	79,90,0	2e-129

BLAST analysis using *R. typhi* (Str. Wilmington) LpxJ primary protein sequence as the query. I%; Identity%, P%; Positives%, G%; Gaps%

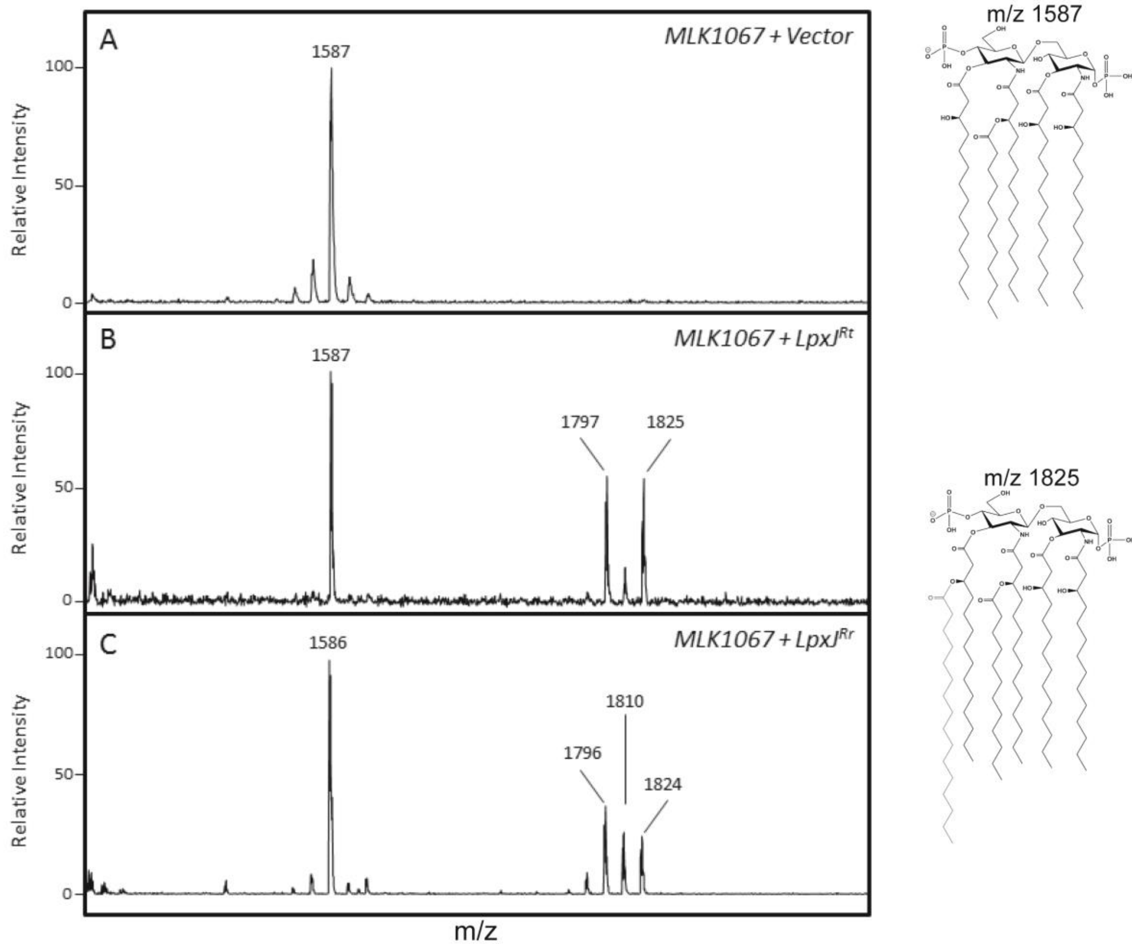


Figure 3.2 LpxJ^{Rt} and LpxJ^{Rr} complement the loss of LpxM in *E. coli* and restore a hexa-acylted lipid A phenotype.

(A) *E. coli* mutant strain MLK1067 (Δ*lpx*M) produces mostly penta-acylated lipid A, corresponding to a major ion peak at m/z 1587. Expression of (B) LpxJ^{Rt} or (C) LpxJ^{Rr} restores a hexa-acylated lipid A phenotype by addition of C14 or C16 fatty acids corresponding to molecular ions at m/z 1797 and m/z 1825, respectively.

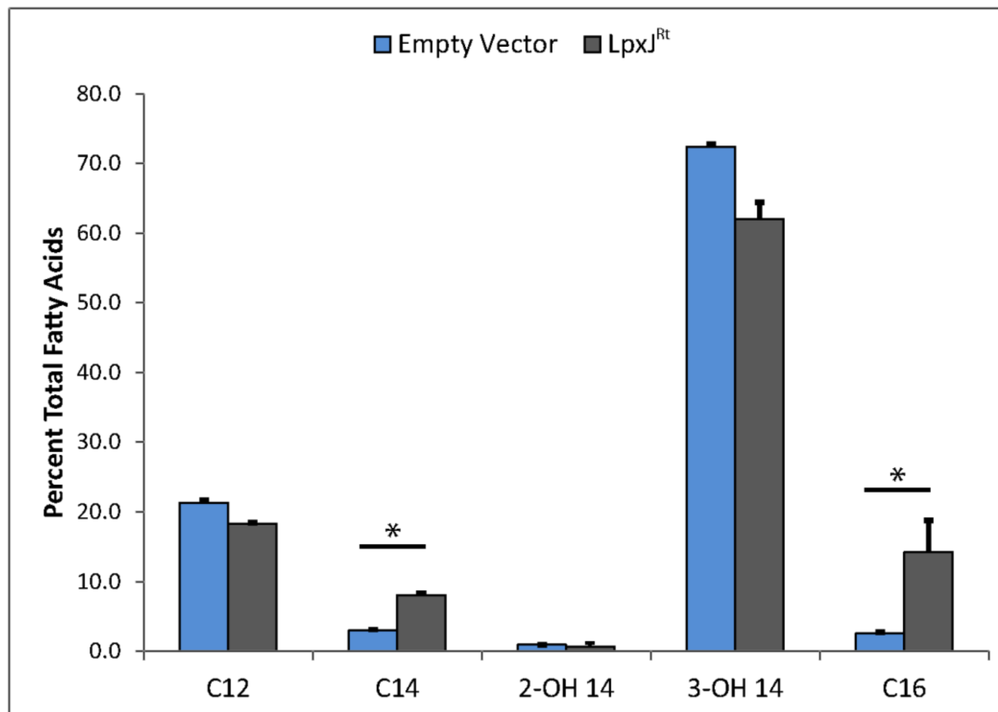


Figure 3.3 LpxJ transfers secondary C14 or C16 to the hydroxymyristate at the 3'- position.
The fatty acid proportions present in the LPS isolated from *E. coli* strain MLK1067 carrying an empty vector (Blue) or expressing lpxJ of *R. typhi* (Grey)given as a percent of the total identified fatty acids. A 4-fold increase of C14 and C16 addition was observed in the *E. coli* mutant complemented with *lpx*J^{Rt}. Analysis was run in biological triplicate and plotted +/- SD. Asterisks indicate statistically significance differences ($p<0.05$) determined by Students t-Test.

LpxJ^{Rt} activity is independent of LpxL activity. In *E. coli* lipid A synthesis, the final fatty acid addition by LpxM requires the prior activity of LpxL, the 2'-secondray acyltransferase (KDO_2-lauroyl-Lipid IV_A substrate) (113). In contrast to LpxM's rigid substrate selection, LpxJ has flexibility in its activity. LpxJ of *H. pylori* can act independently of LpxL activity, while homologs from *C. jejuni* and *W. succinogenes* act exclusively on tetra-acylated substrates. LpxJ^{Rt} is shown above to act upon penta-acylated lipid A molecules (Figure 3.2), but it is unclear whether this is the only lipid A precursor that is a substrate. To determine the requirement of LpxL activity on the activity of LpxJ, we expressed LpxJ in *E. coli* strain MKV15b (114), which produces mostly tetra-acylated lipid A lacking secondary acylation on the 2'- and 3'-fatty acids (Figure 3.4). We found that LpxJ^{Rt} acts upon tetra-acylated substrate (m/z 1404), transferring C14 or C16 fatty acids (m/z 1615 and 1642, respectively). These data suggest that rickettsial lipid A synthesis doesn't share the strict operational order of acyl chain incorporation found in *E. coli*.

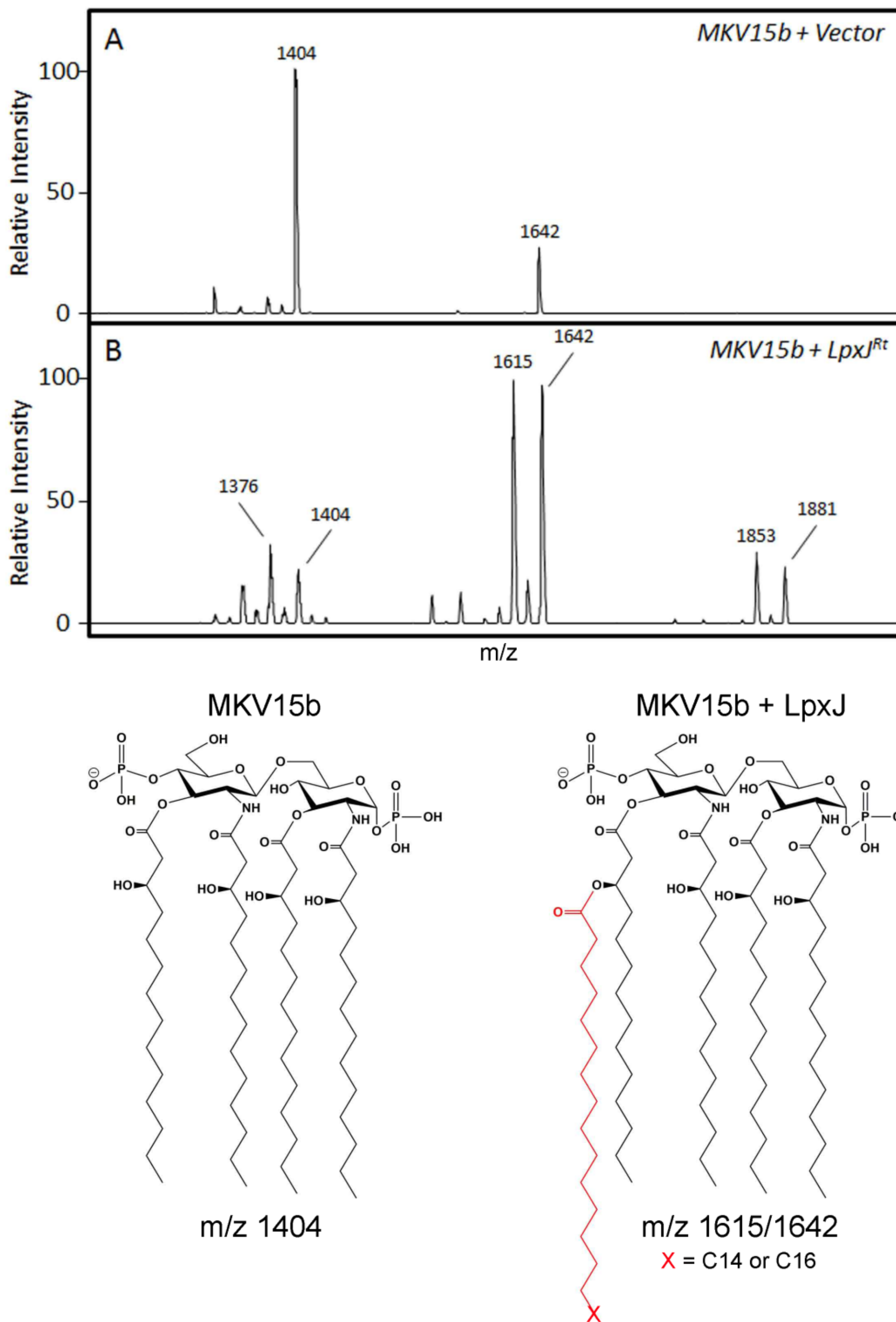


Figure 3.4 Acylation of lipid A by LpxJ^{Rt} does not depend upon prior secondary acylation.
MALDI analysis of lipid A from *E. coli* strain MKV15b (Δ*lpx*M, Δ*lpx*L, Δ*lpx*P, (114)) transformed with (A) Empty vector or expressing (B) LpxJ^{Rt}. The tetra-acylated major lipid A ion of the parental strain (m/z 1404) is acylated by LpxJ which catalyzes the addition of C14 (m/z 1614) or C16 (m/z 1642). The minor ion peak at m/z 1642 in panel [A] is likely the result of minimal constitutive PagP activity in this strain.

Global analysis reveals conserved LpxJ residues critical for acyl transfer. Analysis of 2842 distinct LpxJ homologs identified four conserved regions (Figure 3.5 A). Interestingly, none of these regions contained a HX_4D/E motif, the hallmark acid/base catalytic mechanism, or "charge relay system", that defines GPAT, LPAAT, DHAPAT, and LPEAT acyltransferases (115). To gain insight on residues possibly comprising an alternative charge relay system in the $LpxJ^{Rt}$ active site, we mutated conserved His (H61A, H84A/S) and Asp (D86A, D132A, D173A) residues within these regions, as well two highly conserved Trp residues (W60A, W172A). Aside from H84A/S, these mutants lacked the enzymatic function of $LpxJ^{Rt}$ when expressed in MLK1067, reverting the lipid A phenotype to that of the background strain (penta-acyl, 1587 m/z) (Figure 3.5 B). The negligible effect on $LpxJ^{Rt}$ activity observed by mutating H84 to either Ala or Ser (data not shown) indicates that H61 is the likely catalytic base of LpxJ.

Provided that all three Asp mutants abolished LpxJ activity, we compared LpxJ to four divergent lipid acyltransferases for which structures have been solved (Figure 3.5 C). Modeling $LpxJ^{Rt}$ (RT0047) to these structures consistently positioned H61 within the canonical charge relay system (Block I). Fitting $LpxJ^{Rt}$ to a structural alignment template (116) positioned D132 with other negatively-charged residues in PatA (Block III). This suggests that LpxJ is similar to lipid acyltransferases, which have diverged from the canonical HX_4D/E motif by completing the charge relay system across Blocks I and III (Figure 3.5 C). Unlike PatA however, LpxJ does not have a second positively-charged residue (Block II) coordinating in the active site, which likely explains the lack of a conserved Asp or Glu within Block I for LpxJ. This indicates further divergence of its charge relay system and distinguishes LpxJ from any known lipid acyltransferase.

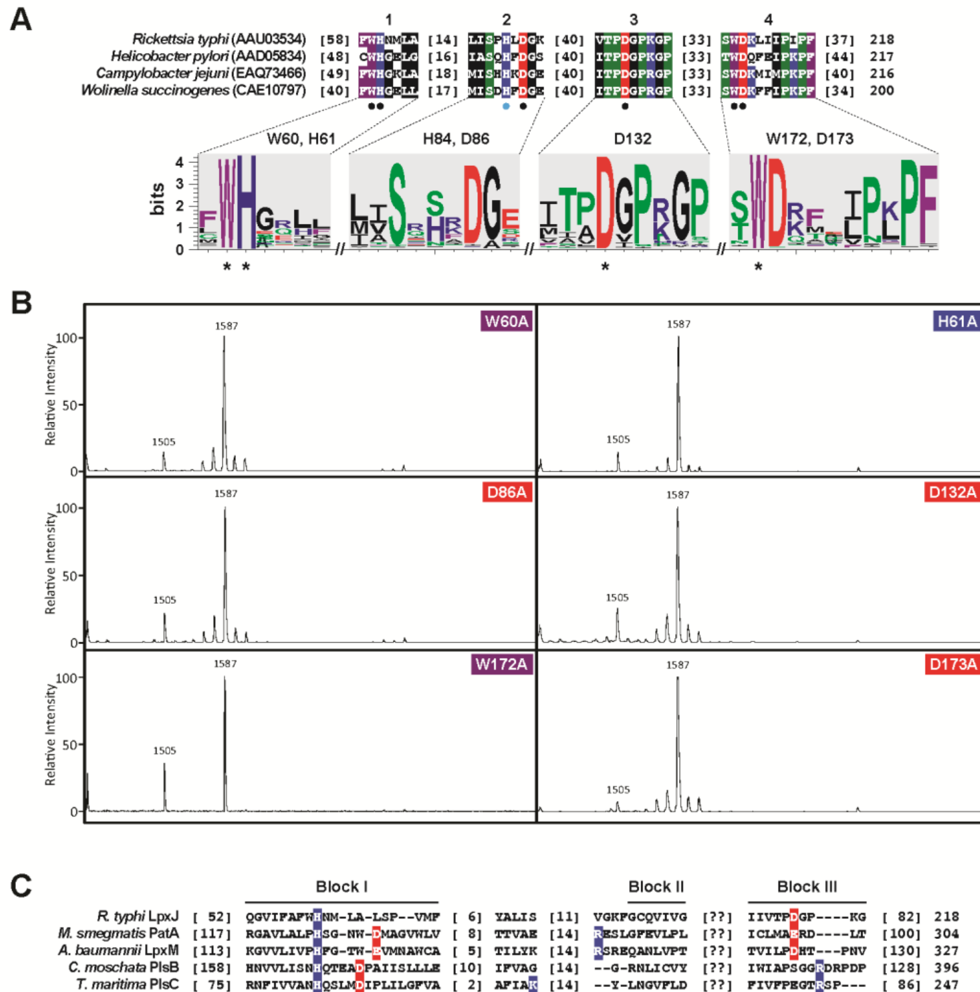


Figure 3.5 Structural and mutational analysis of LpxJ homologs.
(A) Multiple sequence alignment of *Rickettsia typhi* LpxJ and three *Epsilonproteobacteria* LpxJ homologs that were previously characterized as lipid A late acyltransferases (110). NCBI protein accession numbers are in parentheses. Only the four most conserved regions of the alignment are shown (1-4 above). Amino acid coloring as follows: black, hydrophobic; red, negatively charged; green, hydrophilic; purple, aromatic; blue, positively charged. Black and light blue circles below indicate critical and noncritical residues, respectively, as determined by mutagenesis (see panel B). Below each conserved region is a sequence logo (117) depicting conservation across 2842 compiled LpxJ homologs. Asterisks denote the four residues invariant across all LpxJ homologs. (B) The histidine, aspartic acid, or tryptophan residues at the indicated positions in the primary sequence of LpxJ were mutated to alanine and each construct individually expressed in MLK1067. Loss of these highly conserved residues abolished the acyltransferase activity of the enzyme reverting the lipid A phenotype to that of the background strain (penta-acyl, 1587 m/z). Mutation of histidine at position 84 to alanine or serine had no effect on enzymatic activity (not shown). (C) Comparison of *R. typhi* LpxJ to four divergent lipid acyltransferases. Proteins with associated structures were obtained from the PDB: *Mycobacterium smegmatis* PatA (5F34), *Acinetobacter baumannii* LpxM (5KNK), *Cucurbita moschata* PlsB (1IUQ), and *Thermotoga maritima* PlsC (5KYM). *R. typhi* LpxJ was modelled to all four acyltransferase structures using Phyre2 (118) and fitted to an existing structural alignment template (116), which follows the convention established for naming conserved blocks within GPAT, LPAAT, DHAPAT, AND LPEAT acyltransferases (119, 120). Active site residues for each structure are colored. For LpxJ, Asp132 is proposed to participate in the active site charge relay system with His61 (115, 121).

3.4 Discussion

Rickettsia species are obligate intracellular bacterial parasites that produce a typical Gram-negative envelope with the IM and OM separated by a thin peptidoglycan layer (51, 94). Considering the invasive lifestyle of these parasites, the OM sits at the nexus of the host-pathogen interface and is rich with LPS, the classical Gram-negative PAMP (71). Understanding the interplay between mammalian and bacterial molecules at the host-pathogen interface is critically important in development of novel therapeutic approaches. Considering the historical role of LPS as a mediator of inflammation, and its location at the front line of host-pathogen interactions, it is safe to assume that LPS plays a role during *Rickettsia* infection. However, despite its apparent importance, there exists a paucity of information about the biology of LPS biosynthesis and its contribution to virulence. In order to address this gap in our understanding of this molecule, we have begun foundational work elucidating the basics of LPS biogenesis in *Rickettsia*. Here, we characterize new members of the recently discovered LpxJ family of lipid A acyltransferases from *R. typhi* and *R. rickettsii*. Using an *E. coli* reporter system, we have identified $LpxJ^{Rt}$ and $LpxJ^{Rr}$ to be lipid A acyltransferases in *Rickettsia*. These enzymes catalyze the transfer of secondary fatty acids, C14 or C16, to the 3-hydroxyl group of the 3'-primary acyl chain. Interestingly, $LpxJ^{Rt}$ shows no preference for either penta- or tetra-acylated lipid A in our reporter system. This implies that activity of the enzyme is bi-specific for either lipid IV_A or acyl-lipid IV_A and can act before LpxL (RT0704), similar to previously characterized LpxJ homolog from *H. pylori* (110). These data indicate that multiple paths exist for secondary acylation of *Rickettsia* lipid A (Figure 3.6). However,

the requirement for Kdosylation of lipid IV_A prior to $LpxJ^{Rt}$ activity was not tested here. The *E. coli* pathway requires KDO sugars be present on lipid IV_A prior to secondary acylation by LpxL and LpxM (58). However, previously characterized LpxJ homologs did not share this requirement (110). The combination of fatty acid selection (C14-16) and substrate promiscuity (tetra/penta-acyl lipid IV_A) are unique in comparison to the three previously characterized LpxJ homologs, further expanding the diversity of LpxJ-family acyltransferse activity.

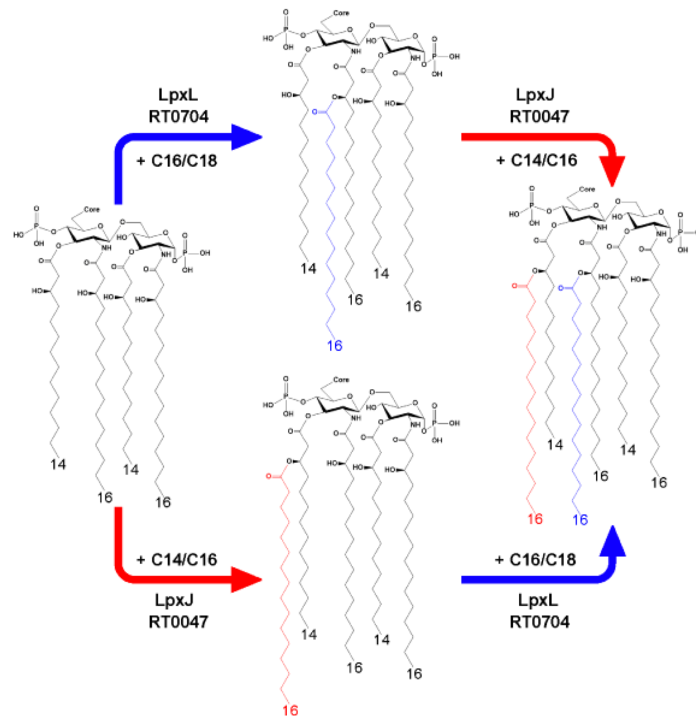


Figure 3.6 Late acyltransferase activity for *R. typhi*.
The bidirectional additions of the final two fatty acids to complete the biosynthesis of lipid A at the conclusion of the Raetz pathway are shown. Blue arrows indicate LpxJ-mediated acylation and red arrows indicate LpxL-mediated acylation.

Our comparative genomics analysis indicates that LpxJ homologs (all members of DUF374) are widespread across Bacteria (Figure 3.5 A). These enzymes have analogous function to the canonical LpxM acyltransferase and their discovery fills a long-standing hole in the Raetz pathway of lipid A biosynthesis in LpxM-deficient bacteria. Indeed, *in silico* structural prediction of $LpxJ^{Rt}$ indicates superficial similarities across LpxJ and PatA, a recently crystalized member of the LPLAT superfamily that contains an active site deviating from canonical lipid acyltransferases (Figure 3.5 C). Further, we identified six highly conserved residues in all LpxJ homologs, which we confirmed were necessary for LpxJ function (Figure 3.5 A,B). These include a histidine at position 61 and an aspartate at position 132 that we believe represent an active site catalytic dyad similar to PatA. Though these amino acids are distant in the primary sequence, structural modeling predicts a close spatial arrangement of these two residues (116, 122) (Figure 3.5 C). It is likely that these residues are positioned sufficiently close to allow charge interactions, abstracting a proton from the acceptor hydroxyl and facilitating nucleophilic attack of the incoming acyl chain thioester bond. This putative charge-relay system is supported by the recently published crystal structures of PatA from *Mycobacterium smegmatis* (116, 122) which does not contain the classical HX_4D motif that is found in other lipid A acyltransferases (123–125) but is absent in LpxJ. Based on the conservation of the proposed catalytic residues within DUF374-containing acyltransferases, this active site orientation might be common within the LpxJ family.

Lipid A architecture is fundamental to bacterial OM integrity. In many Gram-negative pathogens, changes in lipid A structure can have a profound impact on virulence (60, 126). Therefore, we infer that *Rickettsia* LpxJ is also important in maintaining ideal

membrane dynamics and facilitating molecular interactions at the host-pathogen interface that are required for adhesion and invasion of mammalian cells. Additionally, lipid A is the endotoxic component of LPS (63) and activation of TLR4 is critical for bacterial clearance in mouse models of *Rickettsia* infection (108, 127), as well as other intracellular parasites (128–130). These data, as well as a recent report highlighting host-specific differences in lipid A gene expression (131), suggest a critical role for LpxJ in *Rickettsia* virulence, making this enzyme a tempting target for mutational studies *in vivo.* Further work promises to reveal novel insights into *Rickettsia* pathogenesis and contribute greatly to our understanding of rickettsial OM physiology.

3.5 Methods

Recombinant DNA techniques, bacterial strains, and growth conditions. Primers used in this study were obtained from Integrated DNA Technologies (IDT) and are listed in Table 3.2. Genomic DNA was isolated from *R. typhi* grown in Vero76 tissue monolayer as previously described (132). *lpxJ*^{Rt} (RT0047, NCBI accession number AAU03534) was amplified using Q5 polymerase 2x master mix (NEB M0492) and gel purified (Macherey-Nagel 740609.50) before Infusion cloning (Clontech 639649) into *lac* inducible pFLAG-ctc vector (Sigma; discontinued) using HindIII and XhoI restriction sites. Expression of rickettsial genes after induction was verified by immunoblot detection of the C-terminal FLAG epitope tag (Figure 5.3). The resulting construct is designated pFLAG-*lpxJ*^{Rt}. Site directed mutagenesis reactions of selected residues within pFLAG-*lpxJ*^{Rt} were carried out using QuickChange Lightning kit (Agilent #210519). Primers for mutagenesis are listed in Table 3.2.

Table 3.2. Primers used in this study.
Sequences as listed are in the 5' to 3' orientation. Lower case letters represent nucleotides changed in site directed mutagenesis (SDM).

Primer name	Primer sequence (5'-> 3')	Application
LpxJ_to_pFLAG.Fw	ATATCATATGAAGCTTATGCGAAAAGCTCTTAA AAAATTTTTAAAAAATAGTAAATGCT	Cloning
LpxJ_to_pFLAG.Rv	CCCGGGAATTCTCGAccTTTCTTTAAGCTCTCTGT TAAGCTTTTTAATTGT	Cloning
SiteMut_H61toA_top	GGTGTAATCTTTGCATTTTGGgcTAATATGCTTG CCTTAAGTCCC	SDM
SiteMut_H61toA_bottom	GGGACTTAAGGCAAGCATATTAgcCCAAAATGC AAAGATTACACC	SDM
SiteMut_H84toA_top	ATCTATGCTTTAATATCACCAgcTTTAGATGGTA AAATTTTAAAC	SDM
SiteMut_H84toA_bottom	GTTTAAAATTTTACCATCTAAAgcTGGTGATATT AAAGCATAGAT	SDM
SiteMut_D86toA_top	TATCTATGCTTTAATATCACCACATTTAGcTGGT AAAATTTTAAACGCCATAGTAGGGA	SDM
SiteMut_D86toA_bottom	TCCCTACTATGGCGTTTAAAATTTTACCAgCTAA ATGTGGTGATATTAAAGCATAGATA	SDM
SiteMut_D132toA_top	CAAGGTGCAAATATAATAGTTACACCGGcTGGT CCTAAAGGACCTGTATATAAAGTAAA	SDM
SiteMut_D132toA_bottom	TTTACTTTATATACAGGTCCTTTAGGACCAgCCG GTGTAACTATTATATTTGCACCTTG	SDM
SiteMut_D173toA_top	CTTCTAGGTATTTCAGATTAAAAAGTTGGGcTAA ATTAATAATACCAATACCGTTTGGT	SDM
SiteMut_D173toA_bottom	ACCAAACGGTATTGGTATTATTAATTTAgCCCAA CTTTTTAATCTGAAATACCTAGAAG	SDM
SiteMut_W60toA_top	AAATGAACAAGGTGTAATCTTTGCATTTgcGCAT AATATGCTTGCCTTAAGTCCCGTTA	SDM
SiteMut_W60toA_bottom	ATAACGGGACTTAAGGCAAGCATATTATGCgcA AATGCAAAGATTACACCTTGTTCATTT	SDM
SiteMut_W172toA_top	TACTTCTAGGTATTTCAGATTAAAAAGTgcGGAT AAATTAATAATACCAATACCGTTTG	SDM
SiteMut_W172toA_bottom	CAAACGGTATTGGTATTATTAATTTATCCgcACT TTTTAATCTGAAATACCTAGAAGTA	SDM

Bacterial strains used in this study are listed in (Table 3.3). From frozen stocks, *E. coli* were diluted 1:100 after overnight growth into LB broth supplemented with 1mM magnesium and containing the appropriate antibiotics. Diluted cultures were then grown with shaking (225 RPM) at 37°C to mid-log phase. Protein expression was induced by addition of IPTG to a final concentration of 1mM. Induction was carried out at 42°C with shaking (60RPM) for 90 minutes. Whole cultures were harvested by centrifugation at 5,000 RPM for 10 minutes, the supernatants were discarded, and the pellets were either snap frozen in liquid nitrogen before lipid A micro-extraction or lyophilized prior to fatty acid analysis.

Table 3.3. *E. coli* strains used in this study.
CGSC: Cole Genetic Stock Center, Yale.

Strain	Genotype/Description	Reference
MKV15b	*Δlpx*M *Δlpx*L *Δlpx*P	Vorachek-Warren et al., 2002 (114)
MLK1067 (CGSC#7701)	*λ- lpxM11(Ω)::Cm IN(rrnD-rrnE)1 rph-1*	Karow M, and Georgopolous C. 1992 (113)
MLK+LpxJ	MLK1067 transformed with pFLAG vector carrying RT0047 ($LpxJ^{Rt}$)	This work
MLK+JH61A	MLK+LpxJ with His 61 mutated to alanine	This work
MLK+JH84A	MLK+LpxJ with His 84 mutated to alanine	This work
MLK+JW60A	MLK+LpxJ with Trp 60 mutated to alanine	This work
MLK+JD86A	MLK+LpxJ with Asp 86 mutated to alanine	This work
MLK+JD132A	MLK+LpxJ with Asp 132 mutated to alanine	This work
MLK+JW172A	MLK+LpxJ with Trp 172 mutated to alanine	This work
MLK+JD173A	MLK+LpxJ with Asp 173 mutated to alanine	This work

Lipid A micro-extraction. Micro-extraction of lipid A from *E. coli* cultures was performed as previously described (90, 91). Briefly, pellets from 5 ml of mid-log phase *E. coli*, grown and induced as described above, were extracted in 400 µL of a solution containing five parts of isobutyric acid: three parts of 1M ammonium hydroxide and heated at 100°C for 1 h followed by a 15-min incubation on ice and centrifugation at 2000 × g for 15 min. Supernatant was collected and mixed in equal parts with water and then frozen and lyophilized. Contaminants were washed from the dried material by two rounds of methanol washes: 1 ml of methanol, sonicating, and pelleting at 10 000 × g for 5 min. The final product was reconstituted in 2:1:0.25 Chloroform:Methanol:Water (50 µL) along with 4–8 grains of Dowex ion exchange resin (Fisher Scientific, Pittsburgh, PA, USA), incubated with vortexing for at least 5 min. Solubilized lipid A molecules (1-2 µL) were spotted onto a stainless steel target plate along with 1 µL of Norharmane matrix (10mg/ml in 2:1 C:M) for MALDI analysis on a Bruker MicroFlex matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry instrument in negative-ion mode calibrated with Agilent Tuning Mix (Santa Clara, CA, USA. G2421A) and data were processed in flexAnalysis (Bruker Daltonics). All microextraction chemicals were obtained from Sigma-Aldrich unless otherwise noted.

Gas chromatography fatty acid analysis. LPS fatty acids were converted to fatty acid methyl esters (FAMEs) and analyzed using gas chromatography flame ionization detection (GC-FID) as previously described (133). Briefly, lyophilized bacterial cell pellets from 50 ml cultures prepared, as described above were incubated at 70°C for 1 h in 500 µl of 90% phenol and 500 µl of water. Samples were then cooled on ice for 5 min and centrifuged at 9391 × g for 10 min. The aqueous layer was collected and 500 µl of

water was added to the lower (organic) layer and incubated again. This process was repeated two additional times and all aqueous layers were pooled together. Two milliliters of diethyl ether (Fisher E-138-1) was added to the harvested aqueous layers. This mixture was then vortexed and centrifuged at 2095 × g for 5 min. The upper (organic) phase was then aspirated off and 2 ml of diethyl ether was added back to the remaining aqueous phase. The mixture was vortexed and centrifuged at 2095 x g for 5 minutes, and the lower (aqueous) phase was collected then frozen and lyophilized overnight. LPS fatty acids were converted to fatty methyl esters, in the presence of 10 µg pentadecanoic acid (Sigma P-6125) as an internal standard, using 200 µl of 2 M methanolic HCl (Alltech, Lexington, KY) at 90°C for 18 h. Samples were cooled to room temperature and 200 µl of NaCl saturated water was added. Converted fatty methyl esters were then extracted twice with hexane and run on a HP 5890 Series 2 Gas Chromatograph. Retention times were correlated to fatty acids using GC-BAME standards (Matreya, Pleasant Gap, PA. 1114).

***In silico* analysis.** To evaluate it as an LpxJ homolog, we aligned *Rickettsia typhi* RT0047 (NCBI locus tag AAU03534) with previously characterized LpxJ proteins: *Helicobacter pylori* (AAD05834), *Campylobacter jejuni* (EAQ73466) and *Wolinella succinogenes* (CAE10797). To further assess conserved regions of LpxJ proteins, we retrieved 2842 putative homologs from the NCBI non-redundant protein database in blastp searches using RT0047 as the query. Proteins were aligned, with four conserved regions further evaluated for conservation using Weblogo (117). Both abovementioned multiple sequence alignments were constructed using MUSCLE (110) (default parameters). Finally, we compared RT0047 to four divergent lipid acyltransferases that

have associated structures: *Mycobacterium smegmatis* PatA (PDB ID: 5F34), *Acinetobacter baumannii* LpxM (5KNK), *Cucurbita moschata* PlsB (1IUQ), and *Thermotoga maritima* PlsC (5KYM). *R. typhi* LpxJ was modeled to all four acyltransferase structures using Phyre2 (118) and fitted to an existing structural alignment template (116), which follows the convention established for naming conserved blocks within GPAT, LPAAT, DHAPAT, AND LPEAT acyltransferases (119, 120).

CHAPTER 4: SUMMARY

4.1 Findings

LPS makes up most of the outer leaflet of the OM and controls membrane properties such as fluidity and permeability. The rickettsial OM sits at the interface between bacteria and host cell and facilitates the molecular interactions required for adhesion, invasion, and induced phagocytosis. The importance of OM dynamics is apparent, and a comprehensive knowledge of OM biology will further our understanding of the molecular mechanisms of intracellular parasitism.

The molecular pathology of rickettsial disease in humans includes production of inflammatory cytokines, activation of endothelial cells, and extravasation of erythrocytes at foci of infection. Signaling through TLR4/MD2 is responsible, in part, for the robust activation of innate immunity. However, the endotoxic element of rickettsial infection is unknown. The classical TLR4/MD2 agonist is lipid A, but the inflammatory capabilities of rickettsial lipid A remains a mystery. Because lipid A is important to OM physiology, rickettsial lifecycle, and may be involved in disease pathology, this work describes key characteristics of rickettsial lipid A.

In Chapter 2, I present the lipid A structure of *R. rickettsii* and further, compare lipid A form three additional *Rickettsia* species to a previously reported structure. Despite a high conservation in proteins of the lipid A biosynthesis pathway between these species examined, *R. rickettsii* elaborates a lipid A molecule that differs in fatty acid length of the acyloxyacyl chain at the 2’ position, relative to the ‘canonical’ rickettsial lipid A structure of *R. typhi, R. montanensis,* and *R. akari.* My work demonstrates that *R. rickettsii*

incorporates a laurate (C12) at this position, relative to palmitate/stearate (C16/C18) incorporated by other rickettsiae. The consequences of this difference are unknown, but it is conceivable that this phenotype is an adaptation to a lifecycle that includes both ticks and mammals.

The LpxJ-family is a recently described group of acyltransferases with analogous function to the canonical LpxM in the lipid A biosynthesis pathway. Formerly named Domain of Unknown Function 374 (DUF374) containing proteins, these enzymes were first described in 2014 within *Epsilonproteobacteria*. Comparative genomic analysis has indicated that LpxJ homologs are widespread across Bacteria and in Chapter 3, I characterize two new members of this enzyme family from *R. rickettsii* and *R. typhi*. These enzymes catalyze the addition of C14/C16 secondary fatty acids to immature lipid A molecules in a reporter system. This acylation occurs on the 3-hydroxyl group of the 3'-primary acyl chain and shows no preference for tetra- or penta-acyl lipid A precursors as a substrate. This bi-specificity is in contrast to the well characterized analog LpxM, which maintains rigid substrate selectivity for acyl-Lipid $IV_{A.}$ Further, my analysis has identified six highly conserved residues within LpxJ that are required for enzymatic activity, including a putative active site histidine. Other lipid A acyltransferases contain a characteristic HX_4D/E motif that contains the catalytic diad of histidine that is juxtaposed to an acidic residue (D/E), but LpxJ homologs lack this motif. Comparison of LpxJ to the recently crystalized PatA acyltransferase, which also lacks the HX_4D/E motif, has implicated histidine 61 and aspartic acid 132 as forming a catalytic pair as due to their close proximity predicted by structural modeling of LpxJ.

Together, these findings shed light on key features of rickettsial OM biology. By characterizing lipid A structural differences between rickettsiae, we can begin to understand how lipid A divergence changes OM physiology, and how this contributes to the persistence of rickettsiae in nature as well as to the severity of rickettsial disease in humans. Further, lipid A is fundamental to OM integrity and facilitates molecular interactions at the interface between host and rickettsial pathogen. Therefore, a comprehensive understanding of lipid A structure and synthesis is a necessary first step in the development of novel membrane-directed therapeutic interventions for rickettsial disease.

4.2 Ongoing and future work

Prior work within the field of rickettsiology deciphering the molecular determinants of disease burden has focused primarily on proteins, specifically secreted effectors, as predictors of virulence. Our lipid-centric approach takes a novel track in exploring host-pathogen interaction at the molecular level. Ongoing work characterizing lipid A from rickettsiae will continue to focus on differences between pathogens and non-pathogens. Specifically, we will focus on strain-level variation. *R. rickettsii* str. Sheila Smith and *R. prowazekii* str. Breinl cause severe rickettsioses, but also have avirulent counterpart strains. These strains, *R. rickettsii* str. Iowa, *R. rickettsii* str. R, and *R. prowazekii* str. Madrid E, will be compared to their pathogenic siblings in order to elucidate any phenotypic and genotypic differences in lipid A biosynthesis and thereby determine how lipid A structural variation influences rickettsial virulence.

In many Gram-negative pathogens, lipid A is recognized by the host innate immune system through TLR4/MD2 extracellularly, and caspases of the non-canonical inflammasome intracellularly. The lipid A moiety of LPS is a ligand for both sensory systems, and agonistic receptor engagement is dependent upon the shape of the lipid A molecule. Both TLR4/MD2, as well as non-canonical inflammasome (Data not shown), are activated upon infection with *Rickettsia* species, though the contribution of lipid A to this inflammatory signaling is unknown. TLR4/MD2 can also be activated by host-derived DAMPs release from damaged or dying cells. Rickettsiae are deleterious to host cells as a consequence of their metabolic parasitism and intracellular growth, so cellular damage, especially to vascular endothelial cells in humans, is a hallmark of rickettsial disease. It is important to understand the molecular causation of inflammation, specifically TLR4/MD2 activation, in order to rationally design and implement therapeutic solutions to rickettsial diseases while minimizing off-target and adverse effects to patients. Our ongoing work will decipher molecular interactions during rickettsial infection that lead to innate immune engagement. Specifically, we will determine the agonistic capabilities of lipid A from rickettsiae. We hypothesize that rickettsial LPS is a poor agonist for TLR4/MD2. Previous work on lipid A from other Gram-negative pathogens has determined that, in comparison to the canonical lipid A of *E. coli*, longer fatty acid chains reduce the signaling output of the molecule. Our future work will center on the premise that rickettsial lipid A only weakly engages TLR4/MD2. It is possible that ineffectual assembly of the lipid A-TLR4/MD2-receptor complex due to the length of the fatty acids in rickettsial lipid A may disrupt receptor dimerization required for efficient signaling. Instead, considering the cytotoxic nature of rickettsial

parasitism, it is conceivable that DAMPS from infected host cells, also called alarmins, engage the innate immune system and contribute significantly to the inflammatory nature of rickettsial disease. This work promises to fill long-standing gaps in our understanding of rickettsial physiology as well as expand our understanding of innate immunity to intracellular pathogens.

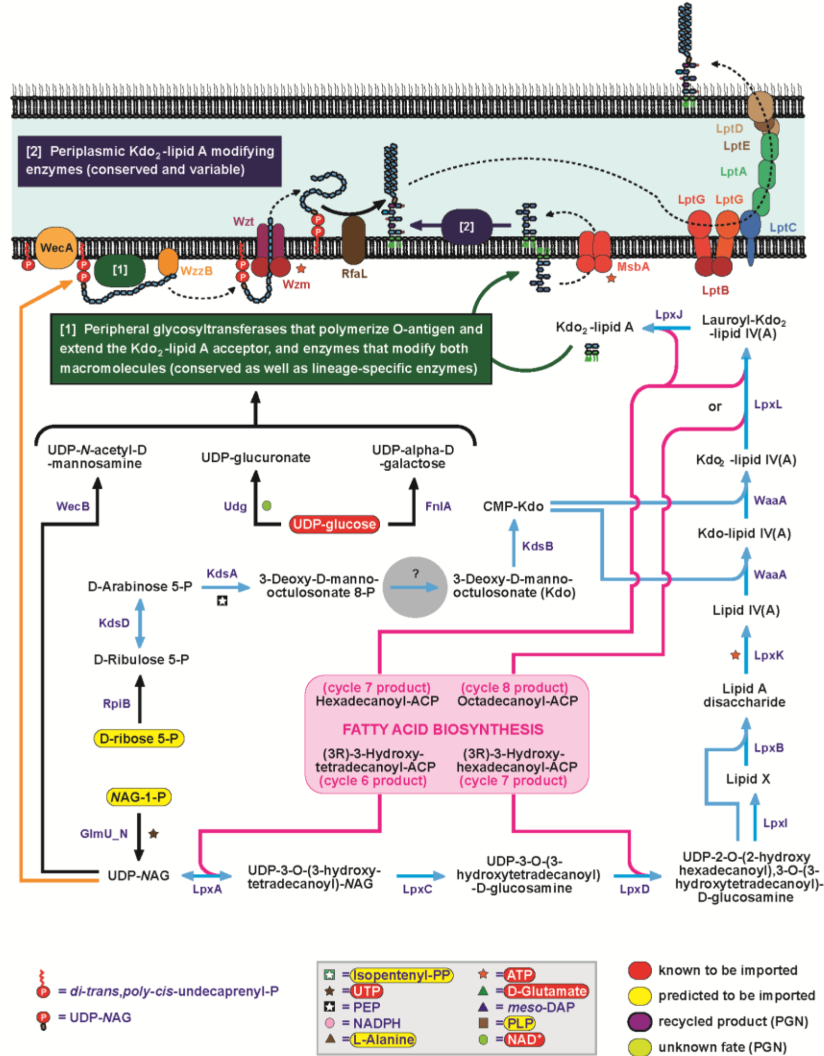


Figure 5.1 Lipopolysaccharide biosynthesis by *Rickettsia* spp. requires import of NAG-1-P, D-ribose 5-P and UDP-glucose from the host.

GlmU_N converts imported NAG-1-P to UDP-NAG, which enters the Raetz pathway (60) for synthesis of Kdo2-lipid A (light blue arrows). Imported D-ribose 5-P is converted to D-ribulose 5-P via ribose-5-phosphate isomerase B (RpiB), leading to synthesis of CMP-Kdo. A pathway hole (gray circle) indicates the absence of a phosphatase to convert 3-deoxy-D-manno-octulosonate 8-P to 3-deoxy-D-manno-octulosonate (Kdo) (4). The incorporation of various fatty acids into the growing lipid A moiety is shown (pink arrows). Acyl chain incorporation into Lipid A follows the structure deduced for *R. typhi* (30). UDP-NAG is also ligated to di-trans,poly-cis-undecaprenyl-P via undecaprenyl-P alpha-N-acetylglucosaminyl 1-P transferase (WecA) (orange arrows), creating the lipid carrier for O-antigen. UDP-N-acetylglucosamine 2-epimerase (WecB) also utilizes UDP-NAG to generate UDP-N-acetyl-D-mannosamine (UDP-ManNAc). Imported UDP-glucose is converted to UDP-glucuronate (UDP-GlcA) and UDP-alpha-D-galactose (UDP-Gal) via UDP-glucose 6-dehydrogenase (Udg) and the epimerase/dehydratase FnlA, respectively. UDP-ManNAc, UDP-GlcA and UDP-Gal are predicted to be the main sugars used by peripheral enzymes (e.g., glycosyltransferases, glucosyltransferases, etc.) that polymerize O-antigen and extend the Kdo2-lipid A acceptor.

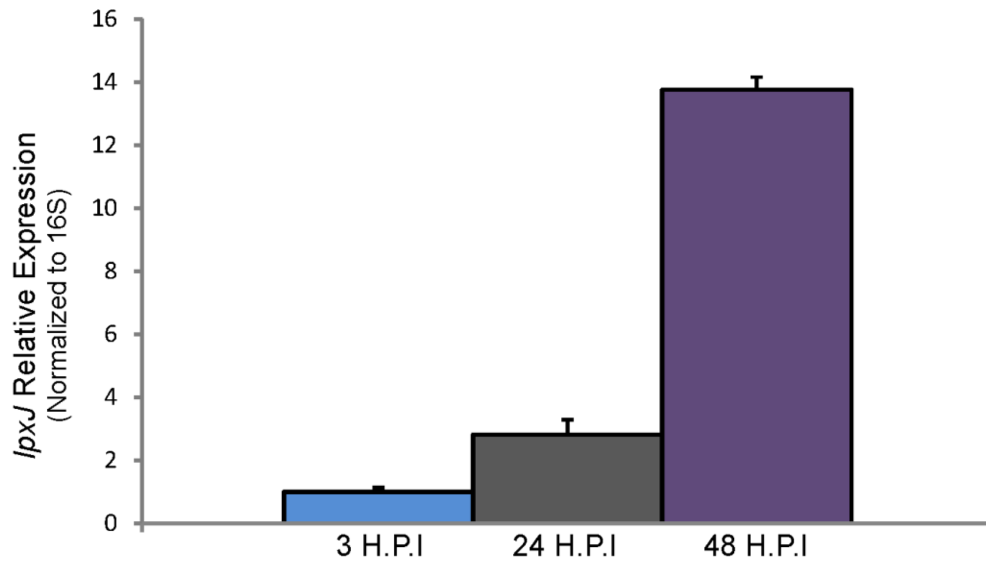


Figure 5.2 lpxJ (Rt0047) is expressed in *R. typhi*.
Vero76 cultures infected with *R. typhi* (MOI ~100:1) were incubated at 34°C for 3, 24, and 48 hours post-infection (H.P.I.). RNA was extracted and the relative level of *lpx*J expression was determined by RT-qPCR using the ΔΔCq method. Data were normalized to expression of the 16s rRNA gene and plotted +/- SEM. 3 H.P.I. time point was set to = 1 and used as a calibrator for the 24 H.P.I and 48 H.P.I samples.

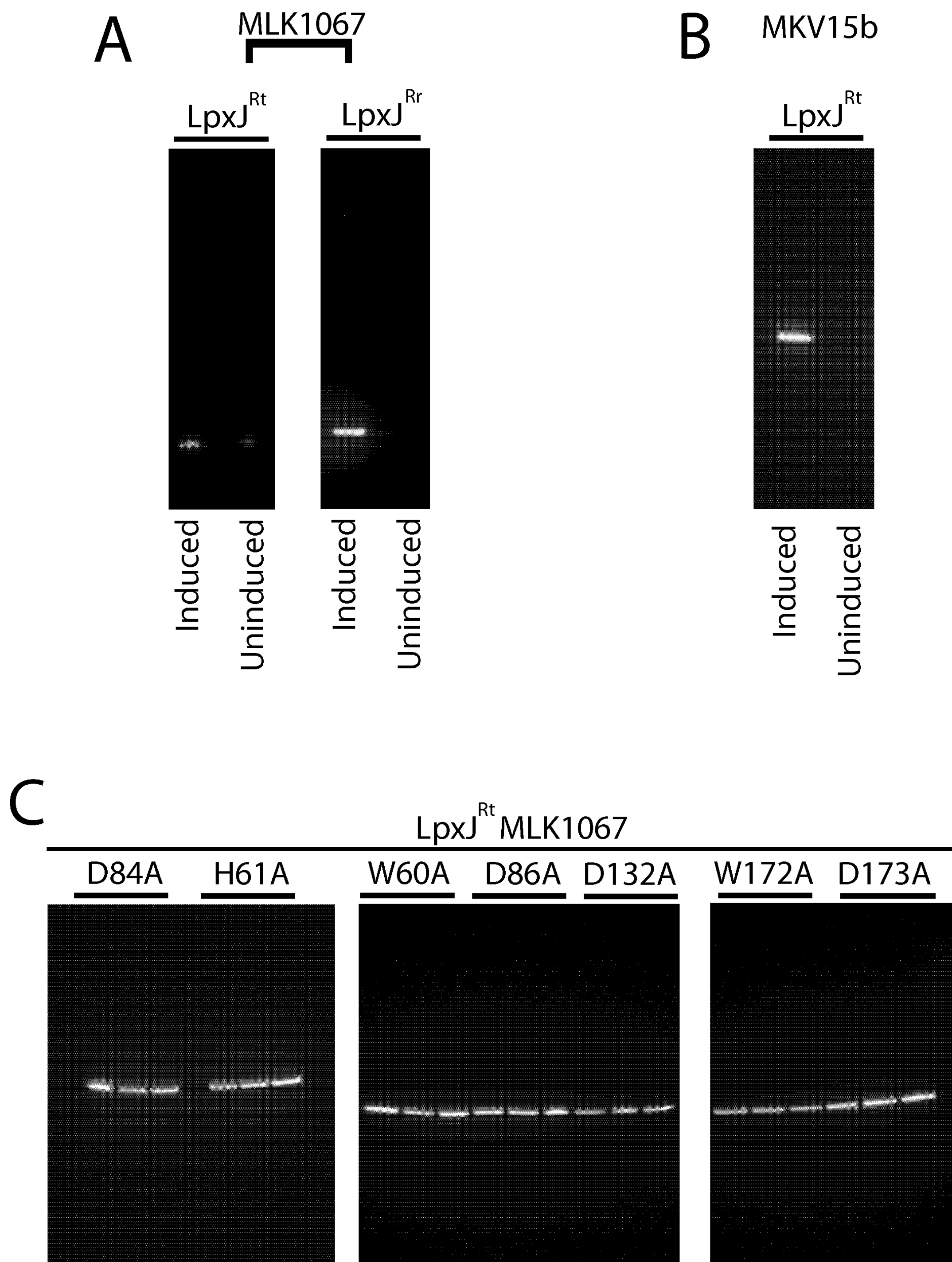


Figure 5.3 LpxJ^{Rt} and LpxJ^{Rr} ectopic expression in *E. coli* mutants.
Whole cellular lysates from *E. coli* strain MLK1067 (A) or MKV15b (B) carrying plasmids encoding the indicated LpxJ homologs were separated by SDS-PAGE and analyzed by immunoblot for the expression of LpxJ using anti-FLAG antibody. (C) Immunoblot detection of LpxJ^{Rt} mutants expressed in MLK1067; 3 colonies each for the indicated point mutation.

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