

## Curriculum Vitae

ALEXIA SMITH

smith.l.alexia@gmail.com | 443 - 771 - 3061

### EDUCATION

**AUG 2022 – MAY 2024** • University of Maryland, Baltimore • Master of Science in Cellular and Molecular Biomedical Sciences

**AUG 2018 – MAY 2022** • Stevenson University • Bachelor of Science in Biochemistry • Minor in Psychology • Departmental Honors

### RESEARCH EXPERIENCE

**FEB 2023 - MAY 2024      Master's Program Thesis Project**

*University of Maryland, Baltimore, HSFI, Baltimore, Maryland.*

Research Rotation under Joe Gillespie, PhD

- Computational research focusing on the transfer of genes to *Rickettsia* and tracing the movement of specific genes through the evolutionary process.

**OCT 2022 - JAN 2023      Master's Program Laboratory Rotation**

*University of Maryland, Baltimore, HSFI, Baltimore, Maryland.*

Research Rotation under Ciaran Skerry, PhD

- Infection experiments using *B.pertussis* on mouse models
- Performed and interpreted ELISA assays.
- Cell culture technique using HEK cells

**JAN 2021 - MAY 2022      Kinetics of Malate Dehydrogenase Inhibition**

*Stevenson University, Owings Mills, Maryland*

Research Assistant under Timothy Dwyer, PhD

- Use of protein column chromatography to isolate MDH proteins.
- Collected and analyzed protein kinetics data from isolated proteins.
- Organized and graphed data.

**MAY 2021 - DEC 2021      The Effect of GPR68 Inhibition on Glioblastoma Survival**

*University of Maryland, Baltimore, Baltimore, Maryland*

Research Assistant under Charles Hong, MD, PhD

- Use of passaging methods to cultivate U87 cells.
- Provided and analyzed data from structural activity relationship studies.
- Use of cell culturing techniques to test varying inhibitors on U87 cells.

**AUG 2021 - DEC 2021      Addition of Ethylene Diamine to Stabilize Cisplatin Analogs**

*Stevenson University, Owings Mills, Maryland*

- Worked closely with partners from other projects to collaborate.
- Created proposals, research plans, and research papers for project.
- Utilized cell culturing methods to test synthesized inhibitor on SKOV3 cells.

**MAY 2021 - AUG 2021      Nathan Schnaper Internship Program**

*University of Maryland, Baltimore, Baltimore, Maryland*

Summer Intern

- Utilized RNA isolation methods, transfection using electroporation and lipofectamine protocols, cell culturing, confocal microscopy, and polymerase chain reaction (PCR).
- Attended lectures hosted by the Marlene and Stewart Greenebaum Cancer Center.

**PUBLICATIONS**

**Stabilization of Cisplatin via Coordination of Ethylenediamine**

*Samantha L. Rea, Alexia Smith, Brooke Hornberger, Grace Fillmore, Jeremy Burkett\*, & Timothy Dwyer. 2022. American Journal of Undergraduate Research. 19(3):37-45.*

[http://www.ajuronline.org/uploads/Volume\\_19\\_3/AJUR\\_Vol\\_19\\_Issue\\_3\\_December\\_2022\\_p37.pdf](http://www.ajuronline.org/uploads/Volume_19_3/AJUR_Vol_19_Issue_3_December_2022_p37.pdf)

**WORK EXPERIENCE**

**AUG 2023      Research Technician**

*University of Maryland, Baltimore, Baltimore, Maryland*

- Maintaining mouse colony.
- Xenogen, Neuromuscular Stimulation, qPCR, and confocal proficiencies

- Aseptic culturing of iPSC cells.

**MAY 2023            Instructor**

*Maryland Science Center, Baltimore, Maryland.*

- Customer Service
- Lead hands-on experiments with students
- Teach Scientific Concepts to 6-13 year olds

**AUG 2022 - AUG 2023        Research Technician**

*University of Maryland, Baltimore, Baltimore, Maryland*

- Husbandry of *Xenopus Laevis*.
- Extraction and Processing of Oocytes.
- Aseptic culturing of iPSC cells.

**MAY 2023            Grill Attendant**

*Diamond Ridge and Woodlands Golf Course, Windsor Mill, Maryland.*

- Customer Service
- Executing and Preparing for Large Events
- Cataloging and Restocking Supplies.

**JUNE 2023    Family Science Night Staff**

*Maryland Science Center, Baltimore, Maryland*

- Lead an experiments with elementary students
- Explain and teach scientific concepts.

**AUG 2019 - MAY 2022        Senior Ambassador**

*Stevenson University, Owings Mills, Maryland*

- Promoted to Senior Leader in May 2020
- Scheduled and trained over 150 employees.
- Marketed the University to prospective students.

**MAY 2021 - MAY 2022        Clinic Assistant**

*MedStar National Rehabilitation Network, Westminster, Maryland*

- Assisting patients through their plan of care
- Organizing files and supplies
- Maintenance and upkeep of machinery.

**MAY 2020 - MAY 2021      Wellness Assistant**

*Asbury Retirement, Solomons Island, Maryland*

- Patient centered care
- Data entry of behaviors and activities of the day.
- COVID-19 response planning in hospice care

**MAR 2019 - AUG 2020      Senior Orientation and Welcome Leader**

*Stevenson University, Owings Mills, Maryland*

- Promoted to senior position in January 2020.
- Scheduled and trained 20 employees.
- Oversee and plan large scale events

**OCT 2020 - DEC 2021      Medical Laboratory Assistant**

*Core Laboratory, Saint Agnes Hospital, Baltimore, Maryland*

- Labelled and entered patients sample tubes into database.
- Data entry and experience with Meditech technology.
- Aseptic technique

**CERTIFICATIONS, ACTIVITIES, AND AFFILIATIONS**

- CPR, AED, First Aid Certification (2017-2023)
  - Phi Sigma Sigma; Zeta Chi Chapter (2020-2022) Vice President and Philanthropy Chair
  - Volunteer Laboratory Assistant (Fall 2019, 2020, 2021)
  - American Chemical Society
  - Pre-Health Student Union (2018-2022) Senior Resident Assistant (2020-2022)
- Dean's List:
- Fall 2018, Fall 2020, Spring 2021, Fall 2021

## ABSTRACT

Title: Evolution of Utilization of Acetate in Pathogenic Rickettsiae

Alexia Smith, Master of Science, 2024

Thesis Directed by: Joseph Gillespie, PhD, Assistant Professor

Rickettsiae are obligate intracellular bacteria with metabolically-reduced genomes. Curiously, only some rickettsiae (i.e. Typhus and Transitional Group pathogens) synthesize large cytoplasmic granules. In unrelated *Proteobacteria*, similar granules contain polyhydroxybutyrate (PHB) as an energy reserve in response to oxidative stress, heavy-metal stress, or excess carbon. PHB chains are degraded to yield substrates for generating cellular energy. It is hypothesized that rickettsiae have conserved the genes for PHB synthesis and utilization. In this study, genes for utilizing acetate (a precursor for PHB biosynthesis) were used to construct a theoretical pathway for PHB synthesis. Phylogenomic analysis was conducted and revealed only Typhus and Transitional Group rickettsiae have conserved the ability to synthesize PHB. Finally, phylogeny estimation of these genes indicates their vertical inheritance from a common *Rickettsia* ancestor, revealing all Spotted Fever Group rickettsiae have lost the ability to utilize acetate. Implications for a role of PHBs in extracellular survival for these rickettsial species are discussed.

Evolution of Acetate Utilization in Pathogenic Rickettsiae

by  
Alexia Smith

Thesis submitted to the Faculty of the Graduate School of the  
University of Maryland, Baltimore in partial fulfillment  
of the requirements for the degree of  
Master of Science  
2024

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## List of Abbreviations

BG	Bellii Group
TG	Typhus Group
TRG	Transitional Group
TIG	Tamurae/Ixodes
SFG	Spotted Fever Group
RMSF	Rocky Mountain Spotted Fever
ATP	Adenosine Triphosphate
Glu	Glutamic Acid
Gln	Glutamine
Asp	Aspartic Acid
PHB	Polyhydroxybuturate
3HB-CoA	3-hydroxybutyryl-CoA
CoA	Coenzyme-A
IG	Intracytosolic Granules

## **Introduction**

Species in the genus *Rickettsia* are Gram-negative coccobacilli bacteria belonging to the Rickettsiales, which is an ancient lineage of *Proteobacteria*.<sup>1</sup> Like all other described members of the family Rickettsiaceae are obligate intracellular and require the intracellular environment for replication.<sup>2</sup> The predominant hosts of *Rickettsia* species are arthropods and protozoa, though environmental sampling indicates a large range of eukaryotic species serve as suitable hosts for rickettsial infection.<sup>3</sup> Many *Rickettsia* species have been categorized into several groups, including a basal lineage called **Bellii Group (BG)** rickettsiae and four later-evolving lineages: **Typhus Group (TG)**, **Transitional Group (TRG)**, **Tamurae/Ixodes Group (TIG)**, and **Spotted Fever Group (SFG)** rickettsiae.<sup>4</sup> Other species remain to be classified into groups, making phylogeny-based taxonomic revision a continual process.

From a human health perspective, the most studied *Rickettsia* species belong to the three lineages that harbor bona fide human pathogens (TG, TRG, and SFG rickettsiae).<sup>5</sup> For example, Rocky Mountain spotted fever (RMSF) is a well-characterized disease caused by *Rickettsia rickettsii* of the SFG rickettsiae. It is contracted by the bite of an infected tick. The presence of this disease is most common in the Rocky Mountain region of the United States. Symptoms include nausea, headache, vomiting, pain in the abdomen, respiratory distress, and potential renal failure.<sup>6</sup> The current fatality rate of RMSF is 10%. The greatest population at risk are people over 40 and under 10 years old. Additionally, people with glucose-6-phosphate dehydrogenase deficiency are at higher risk as well. Other serious human pathogens in the SFG rickettsiae include the agents of Japanese spotted fever (*R. japonica*), Flinders Island spotted fever (*R. honei*), Pacific Coast tick

fever (*R. philipii*), Mediterranean spotted fever (*R. conorii*), Siberian tick typhus (*R. sibirica*), African tick-bite fever (*R. africae*), and Dermacentor-borne necrosis erythema and lymphadenopathy (*R. raoultii* and *R. slovaca*).

Collectively, these SFG pathogens are all transmitted to humans via tick bites, with rickettsia typically infecting small vertebrates in nature. In contrast, TG group pathogens, i.e. the agents of Epidemic Typhus (*R. prowazekii*) and Murine Typhus (*R. typhi*), are transmitted by body lice and fleas with inoculation via insect feces rather than insect feeding.<sup>8</sup> Similar infection dynamics have been described for *R. felis*, a flea-borne TRG pathogen that causes Typhus-like-illness.<sup>9</sup> Less is known about the infection dynamics for other bona fide TRG pathogens, which are transmitted via mites (*R. akari*) and ticks (*R. australis*). Importantly, at least two different modes (blood feeding vs. fecal inoculation) of vertebrate endothelial cell invasion exist for rickettsial pathogens, with insect transmission requiring a longer period in an extracellular state (insect feces).

Bacterium will evolve to have reductive genomes when the metabolites they produced are accessible from the host cytosol as they become intracellular and no longer require the genes to synthesize them.<sup>10</sup> All described rickettsiae lack the ability to synthesize numerous metabolites necessary for growth and proliferation. For example, rickettsiae are not capable of performing glycolysis, requiring host metabolites to serve as precursors for the synthesis of fatty acids, membrane phospholipids, cell wall glycoconjugates, nucleotides, and numerous cofactors.<sup>11</sup> Furthermore, host precursors must fuel the TCA cycle to provide energy (ATP) and metabolic offshoots for oxidative phosphorylation and generation of three amino acids (Gln, Glu, and Asp). All other amino acid synthesis pathways are absent from *Rickettsia* genomes, with specific (yet possibly general)

scavenge mechanisms likely in place for pirating all proteinogenic amino acids from host cytosol. With very little metabolites synthesized by rickettsia, an estimated 51 metabolites are taken from the host cell in a parasitic relationship.<sup>11</sup>

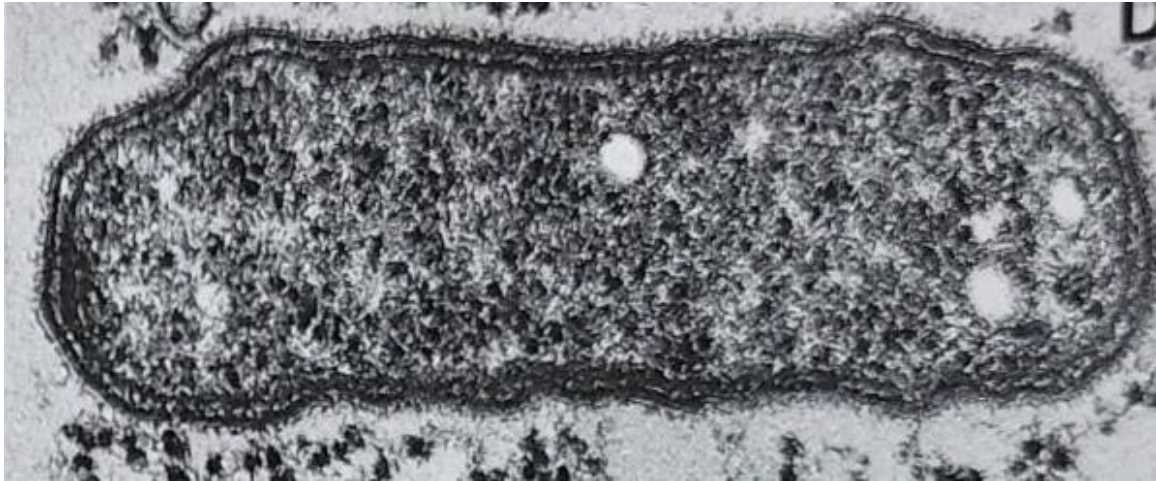


Figure 1. Unpublished photo of *Rickettsia prowazekii* (TG) taken on electron microscope by David Silverman, PhD (used with permission).

When inside a host cell with an abundance of metabolites, some *Rickettsia* species begin to express granules in their cytoplasm that are observable with electron microscopy (Figure 1). These granules are hypothesized to contain polyhydroxybutyrate (PHB) due to the phenotypic similarity to *Cupriavidus necator*. *C. necator* is widely studied for its ability to synthesize and utilize PHB polymers.<sup>12</sup> These polymers allow *C. necator* to store carbon for later use when its environment is no longer carbon rich.

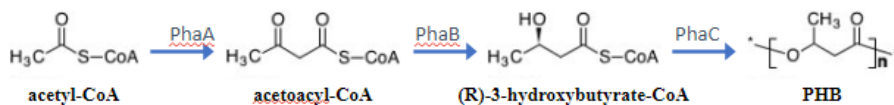


Figure 2. Schematic of the synthesis of polyhydroxybutyrate

PHB is created via carbon assimilation in carbon rich environments as an energy storage mechanism. PHB synthesis may also happen under exogenous stress including excess

hydrogen peroxide, ethanol, and heavy metals.<sup>13-14</sup> The production of PHB in bacteria is conserved across species.<sup>15</sup> The enzymes required in PHB synthesis are 3-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase. These enzymes are encoded by the genes PhbA, PhbB, and PhbC.<sup>12</sup> Due to the conservation of the pathway across bacterial species, the pathway outlined by Peoples and Sinskey was used to begin the metabolic outline in rickettsiae.<sup>16</sup> Beginning with two acetyl-CoA molecules, 3-ketothiolase (encoded by PhbA) will catalyse a condensation reaction to create acetoacetyl-CoA.<sup>12</sup> Acetoacetyl-CoA will then be reduced to 3-hydroxybutyryl-CoA (3HB-CoA) by acetoacetyl-CoA reductase (encoded on PhbB). Finally, 3HB-CoA reacts with PHB synthase (encoded by PhbC) to form PHB. The schematic for the synthesis is a three-step process beginning with acetyl-CoA as seen in figure 2.

Rickettsiae will uptake host acetate and CoAs as their source of carbon for the TCA cycle. Genes encoding proteins AckA and Pta are used to break down acetate into acetyl-CoA (though note the reaction is reversible).<sup>17</sup>

Phasin (PhbP among many other annotations) is an important factor in PHB regulation. Phasins are amphiphilic proteins that will coat PHB in order to stabilize it in the cytoplasm; forming granules.<sup>18</sup> Additionally, phasins are thought to have a role in PHB degradation and to increase the expression of PHB synthases.

During nutrient depletion, granule degradation is primarily carried out by PhbZ (PHB depolymerase), which cleaves PHB into  $\beta$ -hydroxybutyrate monomers to begin the recycling process back to acetyl-CoA for use in the TCA cycle, Fatty Acid synthesis, and generating cellular acetate.<sup>19</sup> While other PHB metabolism-associated genes have been described for different bacteria, the genes needed for synthesis and degradation of PHB

(and involvement of phasins) is well conserved and sufficient to generate observable storage granules via electron microscopy.

We hypothesize that the species of *Rickettsia* with phenotypic granules have conserved the genes needed for PHB synthesis and utilization. In this study, unpublished transcriptomic data was mined for PHB gene expression to construct a metabolic pathway for the synthesis of PHB. Additionally, the species-specific utilization of PHB was analyzed using NCBI's BLAST and aided in estimating phylogenies to understand acetate utilization gene origins.<sup>20</sup> Specifically, my experiments were designed to 1) determine if species with observable cytoplasmic granules also have acetate utilization genes, and 2) assess if acetate utilization genes were gained via lateral genes transfer or secondarily lost in species not known to make storage granules.

## **Materials and Methods**

### ***HaloBlast***

Table 1. Parameters for each HaloBlast query.

Blastp Search	NCBI Taxid	Excluding
<i>“Rickettsia”</i>	780	-
<i>“Rickettsiaceae”</i>	775	<i>“Rickettsia”</i>
<i>“Rickettsiales”</i>	766	<i>“Rickettsia”</i> , <i>“Rickettsiaceae”</i>
<i>“Alphaproteobacteria”</i>	28211	<i>“Rickettsia”</i> , <i>“Rickettsiaceae”</i> , <i>“Rickettsiales”</i>
<i>“Proteobacteria”</i>	1224	<i>“Rickettsia”</i> , <i>“Rickettsiaceae”</i> , <i>“Rickettsiales”</i> , <i>“Alphaproteobacteria”</i>
<i>“Bacteria”</i>	2	<i>“Rickettsia”</i> , <i>“Rickettsiaceae”</i> , <i>“Rickettsiales”</i> , <i>“Alphaproteobacteria”</i> , <i>“Proteobacteria”</i>
<i>“minus bacteria”</i>	6	<i>“Rickettsia”</i> , <i>“Rickettsiaceae”</i> , <i>“Rickettsiales”</i> , <i>“Alphaproteobacteria”</i> , <i>“Proteobacteria”</i> , <i>“Bacteria”</i>

To examine the presence of PHB synthesis and acetate utilization genes and uncover their distribution across species, NCBI BLASTp database was used. The *Rickettsia typhi* proteins PhbA, PhbB, PhbC1, PhbCd, PhbP, PhbZ, Pta, AckA, WecH were used as queries. The method utilized was the HaloBlast method.<sup>19</sup> HaloBlast is the strategy of collecting Blastp subjects using taxonomic databases by sampling lower levels of bacterial classification to observe how they decrease in similarity. The application of HaloBlast in this study included using Blastp searches for each protein against seven different taxonomic databases as laid out in table 1.

Other parameters for each search included 500 max target sequences. The resulting dataset was then used to calculate an *Sm* score using the following equation:

$$Sm = (\text{bitscore of match}) * (\% \text{ aa identity}) * (\% \text{ length of query covered in the match})$$

This score allows for identifying long stretches of similarity and deflating highly similar yet short stretches of similarity.<sup>21</sup> The resulting scores are then compared to each other to find the taxon with the strongest similarity to the individual queries. This dataset was utilized in the phylogeny estimations as well as the species-specific utilization graph.

### ***Species Specific Utilization***

The HaloBlast dataset was used to visualize the species-specific utilization of acetate. The data collected from the '*Rickettsia*' inquiry for all nine proteins highlighted the species with those genes in their genome. They were assigned a color based off the length of the sequence to show whether it was present fully (green), truncated (blue), a pseudogene (black), or not present at all (x).

### ***Metabolic Pathway Construction***

Gene transcripts (unpublished data) were generated using fleas and HeLa cells infected with *Rickettsia typhi*. These transcripts were used to observe upregulated gene discrepancies dependent on host. These differences were used to construct a PHB synthesis metabolic pathway specific to *Rickettsia* species. Additionally, established PHB pathways in other bacteria were used to create the basic structure before exploring the *Rickettsia*-specific differences.

### ***Phylogeny Estimation***

Phylogenetic trees are estimated using the dataset collected from the HaloBlast queries. All *Rickettsiaceae* sequences were included as well as the top five highest scoring *Sm* sequences from Rickettsiales, *Alphaproteobacteria*, *Proteobacteria*, and Bacteria. This will generate trees tracing back to the closest bacterial relation while giving detailed branching as the tree approaches minimal *Rickettsia* divergence. The collected sequences were aligned using Clustal.<sup>22</sup> AliView was used to visualize the alignments before being exported into ATGC PhyML to generate the tree.<sup>23-24</sup> Finally, the tree data was exported into Figtree to visualize and generate a photo of the final phylogeny.<sup>25</sup> This was repeated for all nine genes of interest.

## Results

### Gene Expression of *Rickettsia typhi*

	CDS	Protein (Function)
	RT0282, RT0283	RvhB10, RvhB11 (rvh t4SS)
★	<b>RT0676</b>	<b>PhbZ (PHB)</b>
★	<b>RT0722, RT0723</b>	<b>PhbA (PHB), FadI (Fatty Acid)</b>
	RT0161	AcrB (aminoglycoside efflux)
	RT0674, RT0675	UbiB, UbiE (ubiquinone)
★	<b>RT0026</b>	<b>AckA (acetate)</b>
★	<b>RT0027</b>	<b>Pta (acetate)</b>
	RT0399	Rickettsial conserved HP
	RT0268	HP (in TG and <i>R. felis</i> only)

Overexpressed  
(*C. felis* vs HeLa)

Created in BioRender.com bio

Figure 3. Overexpression of genes in *R. typhi* when infecting *C. felis* versus HeLa cells. The bolded and starred genes are present in PHB synthesis pathways.

Unpublished RNA-seq data was mined to determine if there were genes that are expressed differently during arthropod infection versus vertebrate cell infection.<sup>26</sup> (ex. Figure 3) Relative to HeLa cell infection, it was observed that rickettsia (*R. typhi*) infecting Cat fleas (*Ctenocephalides felis*) overexpressed genes encoding 1) several type 4 secretion system components (*rvh*), 2) the multidrug efflux pump inner membrane subunit AcrB, 3) ubiquinone biosynthesis enzymes UbiB and UbiE, 4) two hypothetical proteins without known functions, and 5) five enzymes related to acetate/acetyl-CoA biology (those encoding FadI, PhbZ, PhbA, AckA, and Pta). The Rvh type 4 secretion system (RvhB) is presumably responsible for translocation of vital substrates (DNA,

proteins) across the cell membrane<sup>27,28</sup>. While other bacterial type 4 secretion systems are also involved with aiding in the structure of surface structures like pili, rickettsiae do not elaborate a pilus with the *rvh* type IV secretion system<sup>29,30</sup>. It isn't clear why the genes encoding RvhB10 and RvhB11 were upregulated during flea infection versus HeLa cell infection, although previous compilation of numerous transcriptomic studies failed to indicate consistent expression patterns for *Rickettsia rvh* genes.<sup>29</sup>

AcrB participates in the macromolecular machine AcrA-AcrB-AcrZ-TolC, which is a drug efflux protein complex with broad substrate specificity that uses the proton motive force to export substrates.<sup>31</sup> Aside from drug efflux, this machine is involved in contact-dependent growth inhibition (CDI), acting downstream of BamA, the receptor for CDI.<sup>32</sup> While only a few rickettsiae have been shown to carry CDI toxin-antidote modules, it isn't clear if any species participates in interbacterial antagonism.<sup>33</sup> Curiously, of the two flea infection-upregulated genes encoding hypothetical proteins, one (*RT0268*) is located adjacent to a gene (*RT0269*) encoding a putative *rvh* effector molecule.<sup>34</sup> This two gene model is found associated with conjugative transposons and plasmids and shows a similar pattern of conservation as the acetate utilization genes described below. Finally, the upregulation during flea infection of two genes encoding ubiquinone biosynthesis enzymes UbiE and UbiB is unclear, as this essential cofactor for oxidative phosphorylation/electron transport is presumably required for rickettsial growth in any intracellular environment.<sup>11</sup>

Five of the twelve genes upregulated in fleas versus HeLa cell infection are associated with acetate/acetyl-CoA biology. PhbZ and PhbA have both been characterized in other bacteria for in PHB biosynthesis. PhbZ is involved with the degradation of PHB. The

overexpression of this gene suggests the utilization of synthesized PHB. It is unclear why *R. typhi* is expressing and utilizing PHB during the infection. This raises potential differences in how rickettsiae may utilize PHB differently than another PHB-producing bacterium. The gene *fadI*, encoding 3-ketoacyl-CoA thiolase, catalyzes the final step of fatty acid oxidation in which acetyl-CoA is released and the CoA ester of a fatty acid two carbons shorter is formed. FadI and PhbA are tandemly encoded in most rickettsial genomes (when *phbA* is conserved), indicating a possible coregulation of fatty acid degradation and Acetyl-CoA utilization. Acetate kinase (AckA) and phosphotransacetylase (Pta) are both involved in known acetate utilization pathways. The 12 proteins identified here require further investigation due to their therapeutic potential as drug targets. Overall, the data highlighted here demonstrates that upon infection of the Cat flea, *R. typhi* upregulate genes associated with proliferation indicated by the increased activity of important pathways such as the electron transport chain and transportations across the membranes. Further studies are needed to characterize the hypothetical proteins and to explore the specific effects of the genes in the flea-*R. typhi* interaction.

### Reconstructed Metabolic Pathway for Acetate Utilization

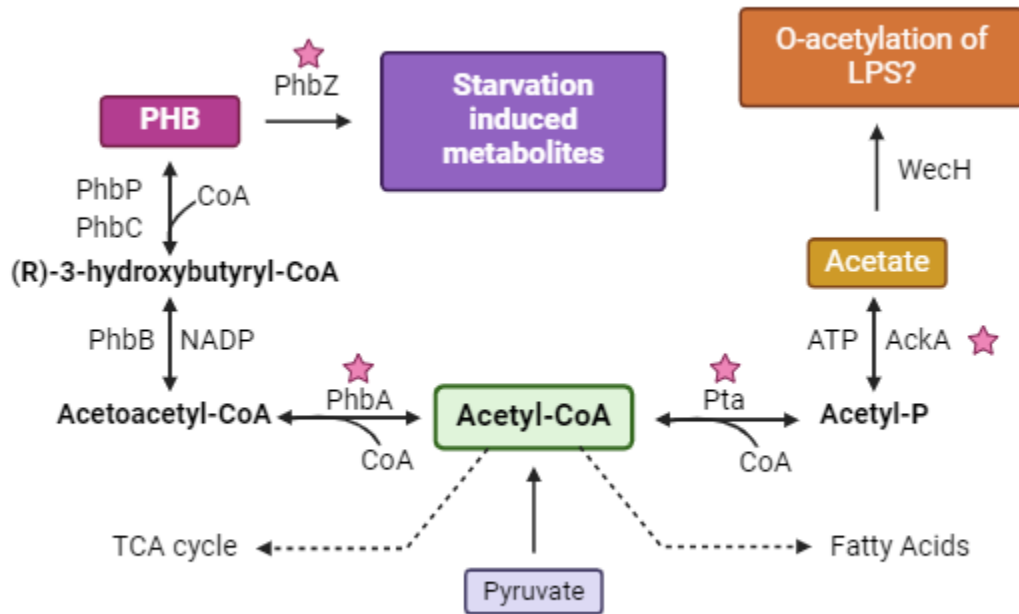


Figure 4. Reconstructed pathway of PHB synthesis and converging acetate synthesis pathways in rickettsiae using existing pathway models and RNA sequencing data from rickettsia-*C. felis* infection models. Purple stars indicate the genes upregulated in figure 3.

The pathway of PHB synthesis and utilization was reconstructed to outline *Rickettsia* specific utilization. The hypothesized pathway was constructed using established PHB metabolic pathways and the RNA sequencing data of the upregulated genes in infected *C. felis*. (ex. Figure 4) This pathway outlines the conversion of acetyl-CoA to PHB and acetate. The ability for the reaction to move in both directions is noted by double-tipped arrows. The pathway starts with acetyl-CoA. Acetyl-CoA and another CoA molecule are condensed by  $\beta$ -ketothiolase to form acetoacetyl-CoA. Then, acetoacetyl-CoA reductase reduces acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA with the help of NADP. Poly-beta-hydroxybutyrate synthase (PhbC) along with phasin (PhbP) synthesizes PHB. When

in a nutrient sparse environment, polyhydroxyalkanoate depolymerase (PhbZ) begins the degradation of PHB back into acetyl-CoA for energy use. This is consistent with known PHB synthesis pathways. On the other side of the pathway, there is a reversible acetate pathway. Starting with an acetate molecule Rickettsia can obtain from their host, AckA and ATP can phosphorylate acetate into acetyl-P. Pta will then condense acetyl-P and CoA into acetyl-CoA. This would allow rickettsia to take acetate as it needs to proliferate as well as begin to store some in the form of PHB for later use. There is also an additional step involving WecH (O-acetyltransferase). The presence of this gene indicates the potential ability of rickettsiae to O-acetylate their lipopolysaccharide. This conformational change will impact the bacterium-host interaction.<sup>35</sup> Further studies are needed to determine the function of WecH in Rickettsial species with the current hypothesized function of this protein is the o-acetylation of LPS molecules.

## Rickettsia Species Specific Utilization of PHB genes

		● full length	● pseudogene	● truncated	× absent	PhaA	PhaB	PhaC1	PhaC2	PhaP	PhaZ	AckA	Pta	WechH
	Endosymbiont ( <i>Bemisia tabaci</i> )	●	●	●	×	●	●	●	×	●	●	×	●	×
	<i>R. bellii</i> * [4]	●	●	●	×	●	●	●	●	●	●	●	●	●
	<i>R. canadensis</i> * [2]	×	×	×	×	×	×	×	×	×	×	×	×	×
	<i>R. helvetica</i> str. C9P9	×	×	×	×	×	×	×	×	×	×	×	×	×
Transitional Group	<i>R. akari</i> str. Hartford	●	●	●	×	●	●	●	●	●	●	●	●	●
	<i>R. australis</i> * [2]	●	●	●	×	●	●	●	●	●	●	●	●	●
	<i>R. hoogstraalii</i> * [2]	●	●	●	×	●	●	●	●	●	●	●	●	●
	" <i>Candidatus R. asemboensis</i> "	●	×	●	●	●	●	●	●	●	●	×	●	×
Typhus Group	<i>R. felis</i> * [4]	●	●	●	×	●	●	●	●	●	●	●	●	●
	<i>R. typhi</i> * [3]	●	●	●	×	●	●	●	●	●	●	●	●	●
	<i>R. prowazekii</i> * [12]	●	●	●	×	●	●	●	●	●	●	●	●	●
	<i>R. tamurae</i> str. AT-1	●	●	●	×	●	●	●	×	●	●	×	●	×
	<i>R. monacensis</i> str. IrR/Munich	●	×	●	×	●	●	●	●	●	●	●	●	×
	Endosymbiont ( <i>Ixodes pacificus</i> )	●	×	●	×	●	●	●	●	●	●	●	●	×
	<i>R. buchneri</i> * [2]	×	●	×	×	×	×	×	×	×	×	×	×	×
	<i>R. montanensis</i> str. OSU 85-930	●	●	×	×	●	●	×	×	×	×	×	×	×
	<i>R. raoultii</i> str. Khabarovsk	×	●	●	●	●	●	●	●	●	●	×	×	×
	<i>R. amblyommatis</i> * [4]	●	●	●	×	●	●	●	●	●	●	×	×	×
Spotted Fever Group	<i>R. aeschlimannii</i> str. MC16	×	×	×	×	×	×	×	×	×	×	×	×	×
	<i>R. rhipicephali</i> * [3]	●	×	×	×	●	●	●	●	●	●	●	●	×
	<i>R. massiliae</i> * [2]	×	×	×	×	●	●	×	●	●	×	×	×	×
	" <i>Candidatus R. gravesii</i> " str. BWI-1	×	×	×	×	×	×	×	×	×	×	×	×	×
	<i>R. japonica</i> str. YH	●	●	●	×	●	●	●	●	●	●	●	●	●
	<i>R. heilongjiangensis</i> str. 054	●	×	●	●	●	●	●	●	●	●	×	×	×
	<i>R. argasii</i> str. T170-B	●	●	×	●	●	●	●	●	●	●	●	●	×
	<i>R. honei</i> str. RB	●	●	●	×	●	●	●	●	●	●	×	×	×
	<i>R. peacockii</i> str. Rustic	×	×	×	×	×	×	×	×	×	×	×	×	×
	Endosymbiont ( <i>Proechinophthirus fluctus</i> )	×	●	●	●	×	×	×	×	×	×	×	×	×
	<i>R. philipii</i> str. 364D	×	●	●	●	●	●	●	●	●	●	●	×	×
	<i>R. rickettsii</i> * [10]	×	●	●	●	●	●	●	●	●	●	×	×	×
<i>R. slovaca</i> * [2]	●	×	●	×	×	×	×	×	×	×	×	×	×	
<i>R. conorii</i> * [4]	●	●	●	●	●	●	●	●	●	●	●	●	×	
<i>R. africae</i> str. ESF-5	●	●	×	×	●	×	×	×	×	×	×	×	×	
<i>R. sibirica</i> * [3]	●	●	●	●	●	●	●	●	●	●	●	●	×	
<i>R. parkeri</i> * [4]	●	●	●	●	●	●	●	●	●	●	●	●	×	

Figure 5. Species-Specific Utilization of the nine genes of interest in the PHB metabolic pathway and acetate utilization pathway using data collected from HaloBlast protocol.

The rickettsial species specific utilization was investigated to discover which species of *Rickettsia* has retained the 9 genes of interest. (ex. Figure 5) This was done by mining the data from the HaloBlast protocol. The majority of TRG rickettsiae have full length acetate utilization genes except for *wecH*. This gene encodes a membrane protein known to O-acetylate enterobacterial common antigen in *Escherichia coli*.<sup>36</sup> We suspect TG rickettsiae use *WechH* to O-acetylate LPS since *wecH* clusters with LPS genes (data not shown). There are a few truncated open reading frames, pseudogenes, and absent genes sporadically present, which is a standard observation with *Rickettsia* comparative

genomics. As for the two typhus group species, they have a fully conserved PHB synthesis gene presence as well as the acetate synthesis pathway. The spotted fever group has very few conserved and full-length sequences of these genes. The most conserved gene in this group is PhbA. We conclude that TG and TRG species of Rickettsia can produce PHB. The SFG seems to have some genes retained, but unable to complete the process. These genes are on their way out of the SFG genome. Additionally, BG rickettsiae also have conserved the ability to synthesize PHB. These differences most likely contribute to the virulence of these separate groups of rickettsiae.

### Phylogenetic Trees for Acetate Utilization Genes

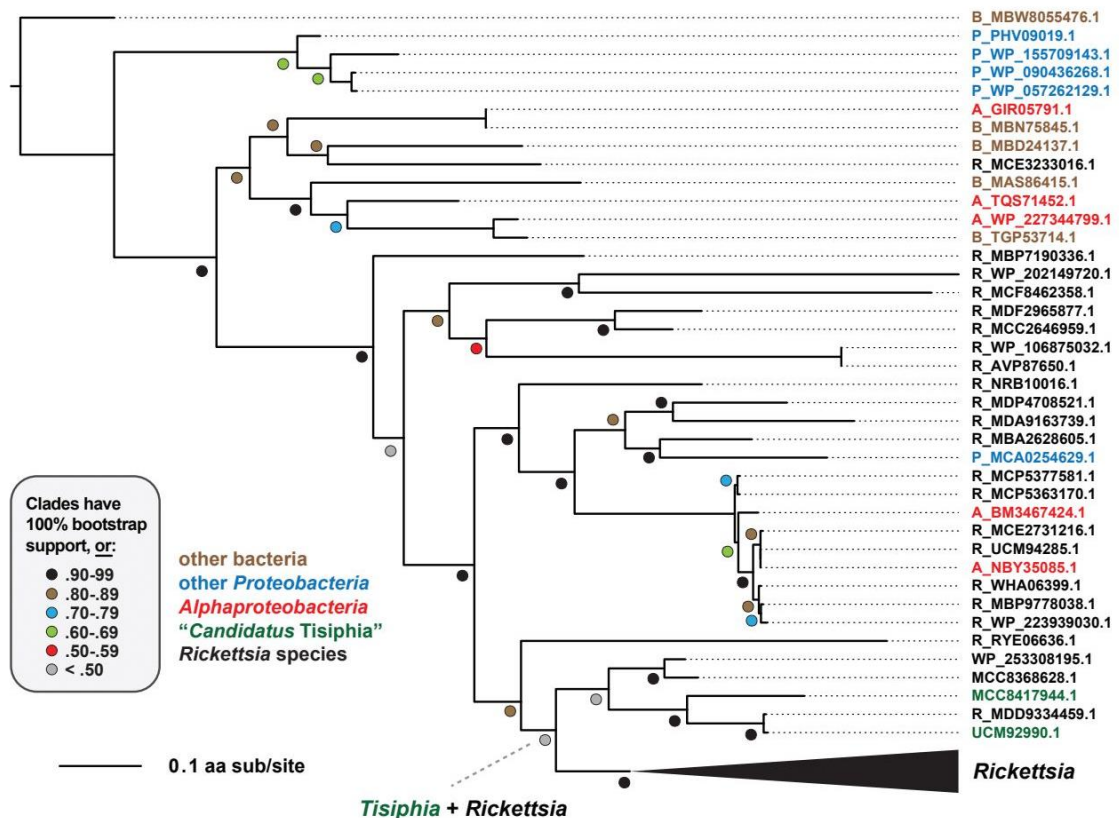


Figure 6. Phylogenetic Tree showcasing the vertical transfer of PhbA gene through *Rickettsia* evolution. Accession numbers are present at the end of each branch correspond with NCBI GenBank accession numbers. Accession numbers in brown indicate an unrelated bacterium. Blue represents *proteobacteria*, red represents *alphaproteobacteria*, green represents "*Candidatus Tisiphia*" and black represents *Rickettsia* species. Bootstrap key has .90-.99 confidence in black, .80-.89 in brown, .70-.79 in blue, .60-.69 in green, 0.50-0.59 in red, and anything less than 0.50 has light gray.

The phylogeny of PhbA gene was estimated to determine the mode of transmission through Rickettsial evolution. *Nitrospora* was used to root the tree due to it being the farthest relation from Rickettsiae species with this gene. The evolution of PhbA is visualized as it passed down through evolution vertically. (ex. Figure 6) The vertical transfer of this gene indicates the conservation of PhbA in BG, TG, and TRG rickettsiae through their evolution. Additionally, the SFG species present (*R. tamurae*, *R. monacensis*, *R. amblyommatis*, *R. montanansis*, *R. slovacae*, *R. honei*, *R. conorii*, *R. parkeri*, *R. africae*) in the tree have the PhbA gene present either completely or truncated (ex. Figure 5). This is further support for the conclusion drawn in the species-specific utilization analysis that PhbA has been conserved for its ability to catalyze the reaction to turn acetyl-CoA into acetoacetyl-CoA and aides in further conclusions that it has been conserved vertically through evolution.

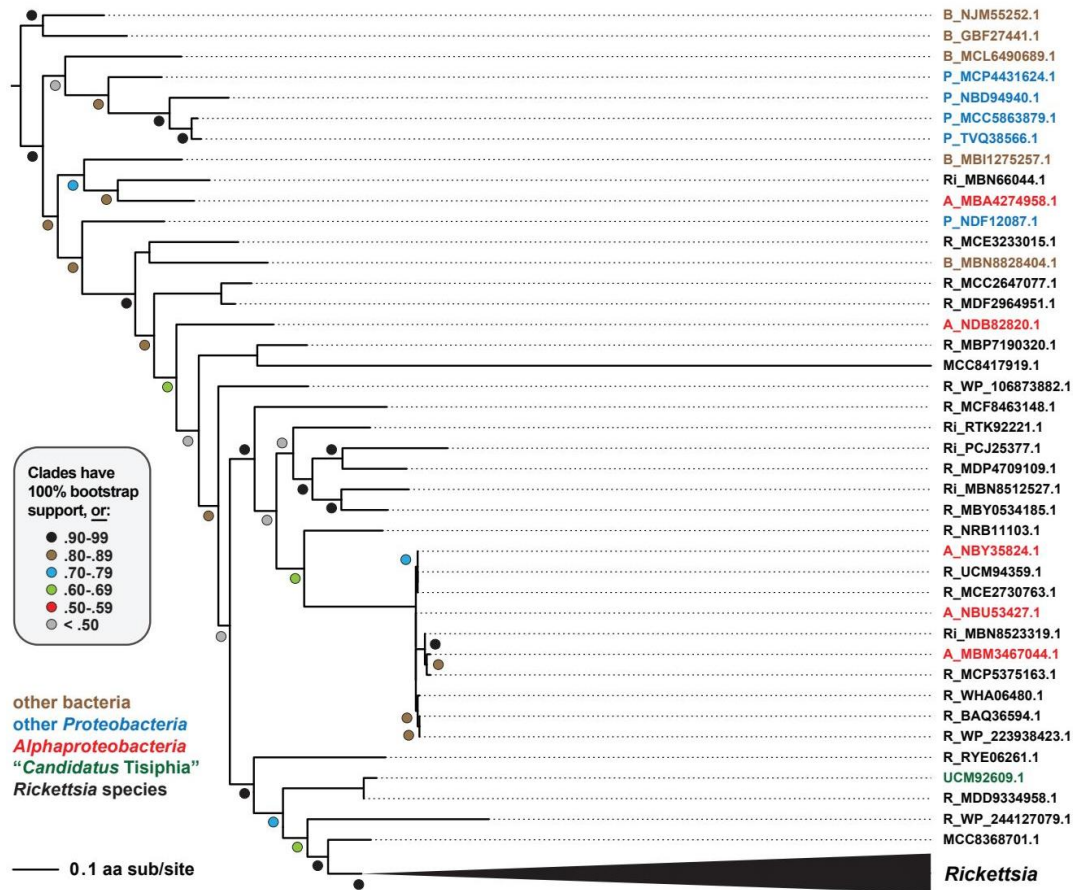


Figure 7. Phylogenetic Tree for the evolution of PhbB in Rickettsiae. Accession numbers are present at the end of each branch correspond with NCBI GenBank accession numbers. Accession numbers in brown indicate an unrelated bacterium. Blue represents proteobacteria, red represents alphaproteobacteria, green represents "Candidatus Tisiphia" and black represents Rickettsia species. Bootstrap key has .90-.99 confidence in black, .80-.89 in brown, .70-.79 in blue, .60-.69 in green, 0.50-0.59 in red, and anything less than 0.50 has light gray.

The phylogeny of PhbB gene was estimated to determine the mode of transmission through Rickettsial evolution. *Verrucomicrobiae* was used to root the tree due to it being the farthest relation from Rickettsiae species with this gene. The evolution of PhbB is visualized as it passed down through evolution vertically. (ex. Figure 7)

Just like PhbA, the vertical transfer of this gene indicates the conservation of PhbB in BG, TG, and TRG rickettsiae through their evolution. Less SFG species can synthesize PhbB with only *R. tamurae*, *R. honei*, *R. conorii*, *R. parkeri*, *R. africae*, and *R. sibirica* present. The decrease of SFG species being able to perform the next step of the PHB

synthesis supports the idea that the PHB synthesis genes are being phased out of the SFG due to it no longer being metabolically essential to their proliferation. However, all the TG and TRG bacteria have conserved this ability as they have evolved.

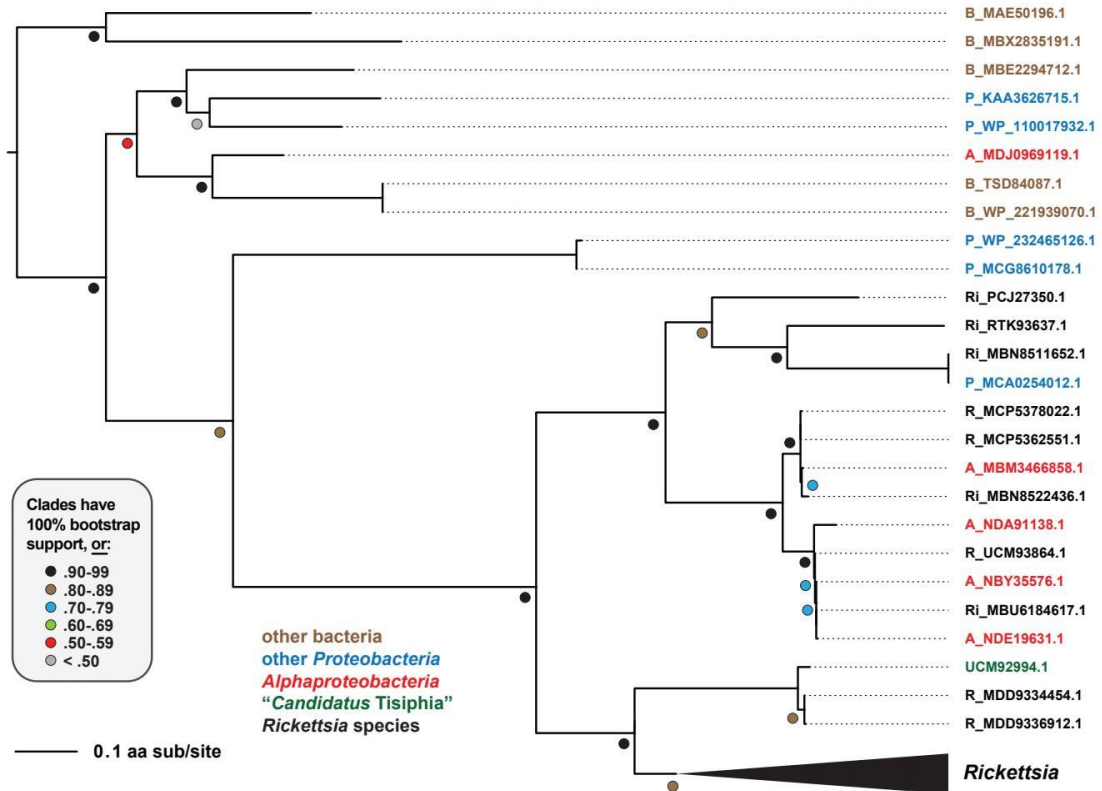


Figure 8. Phylogenetic Tree for the evolution of PhbC in Rickettsiae. Accession numbers are present at the end of each branch correspond with NCBI GenBank accession numbers. Accession numbers in brown indicate an unrelated bacterium. Blue represents proteobacteria, red represents alphaproteobacteria, green represents "Candidatus Tisiphia" and black represents Rickettsia species. Bootstrap key has .90-.99 confidence in black, .80-.89 in brown, .70-.79 in blue, .60-.69 in green, 0.50-0.59 in red, and anything less than 0.50 has light gray.

We estimated the phylogeny of PhbC gene to determine the mode of transmission through Rickettsial evolution. *Micavibrio* was used to root the tree due to it being the farthest relation from Rickettsiae species with this gene. The evolution of PhbC is visualized as it passed down through evolution vertically. (ex. Figure 8)

Once again, the vertical transfer of this gene indicates the conservation of PhbC in BG, TG, and TRG rickettsiae through their evolution. A stark difference for PhbC is the

absence of SFG species. This gene is seemingly not present within the SFG. This indicates that SFG species like *R. conorii*, *R. parkeri*, and *R. honei* are only able to turn acetyl-CoA into R-3-hydroxybutyryl-CoA but lack the final genes needed to synthesize the final PHB molecule. PhbC is present in TG and TRG species further supporting their ability to synthesize PHB was conserved through vertical inheritance.

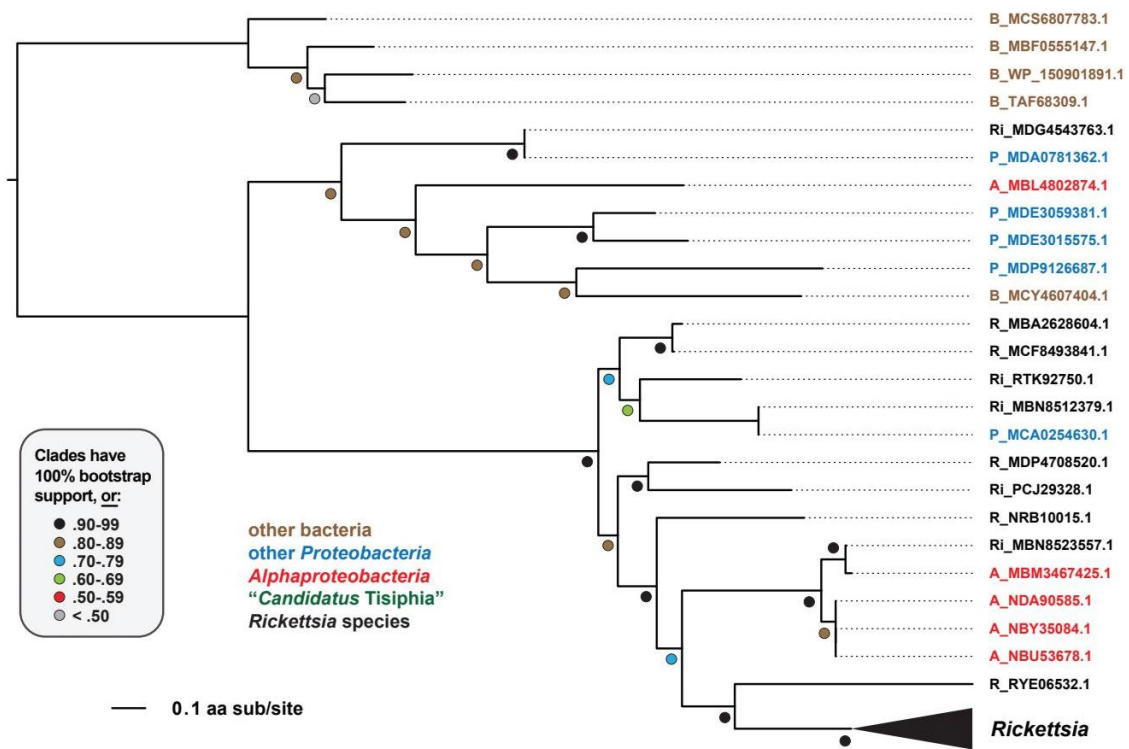


Figure 9. Phylogenetic Tree for the evolution of PhbCd in Rickettsiae. Accession numbers are present at the end of each branch correspond with NCBI GenBank accession numbers. Accession numbers in brown indicate an unrelated bacterium. Blue represents *proteobacteria*, red represents *alphaproteobacteria*, green represents "*Candidatus Tisiphia*" and black represents *Rickettsia* species. Bootstrap key has .90-.99 confidence in black, .80-.89 in brown, .70-.79 in blue, .60-.69 in green, 0.50-0.59 in red, and anything less than 0.50 has light gray.

We estimated the phylogeny of PhbCd gene to determine the mode of transmission through Rickettsial evolution. *Candidatus Kapabacteria* was used to root the tree due to

it being the farthest relation from Rickettsiae species with this gene. The evolution of PhbCd is visualized as it passed down through evolution vertically. (ex. Figure 9)

PhbCd is a player in the PHB pathway without a fully understood role. Some potential explanations include PhbCd being a variant of PhbC or an additional enzyme needed in the formation of granules. This gene is present in TG, TRG, BG, and SFG species. This gene also follows a vertical transfer pattern. This gene is present in *R. conorii*, *R. monacensis*, and *R. rhipicephali* species of SFG rickettsia. Characterization of this gene is needed to determine its role in PHB synthesis, but it is also being phased out of the SFG genome at a similar rate as the other PHB genes.

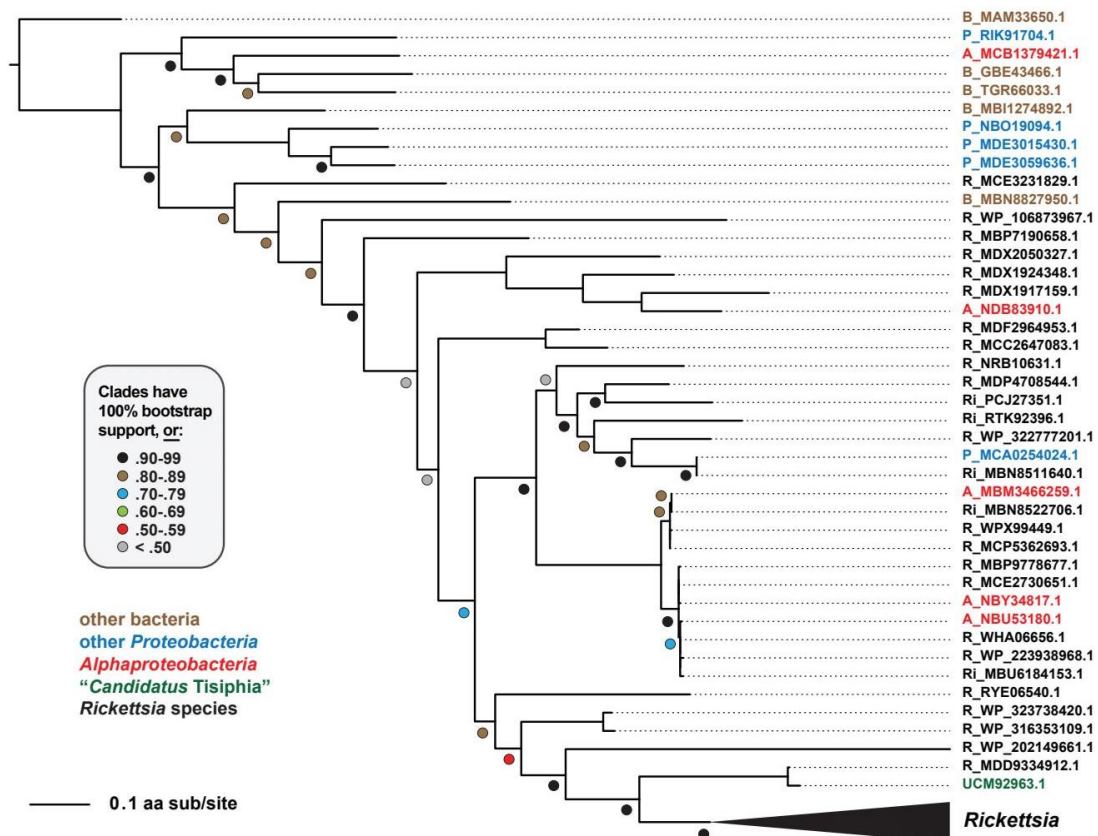


Figure 10. Phylogenetic Tree for the evolution of PhbZ in Rickettsiae. Accession numbers are present at the end of each branch correspond with NCBI GenBank accession numbers. Accession numbers in brown indicate an unrelated bacterium. Blue represents proteobacteria, red represents alphaproteobacteria, green represents "Candidatus

*Tisiphia*” and black represents *Rickettsia* species. Bootstrap key has .90-.99 confidence in black, .80-.89 in brown, .70-.79 in blue, .60-.69 in green, 0.50-0.59 in red, and anything less than 0.50 has light gray.

We estimated the phylogeny of PhbZ gene to determine the mode of transmission through Rickettsial evolution. *Microvibrio* was used to root the tree due to it being the farthest relation from Rickettsiae species with this gene. The evolution of PhbZ is visualized as it passed down through evolution vertically. (ex. Figure 10)

PhbZ encodes for the PHB depolymerase. It is present in TG, TRG, and BG species. As expected, it is not present in any species of the SFG. The gene was conserved in the species that produce PHB and would therefore require PhbZ to degrade the molecule into usable metabolites. The pattern of conservation is a vertical transfer through their evolution.

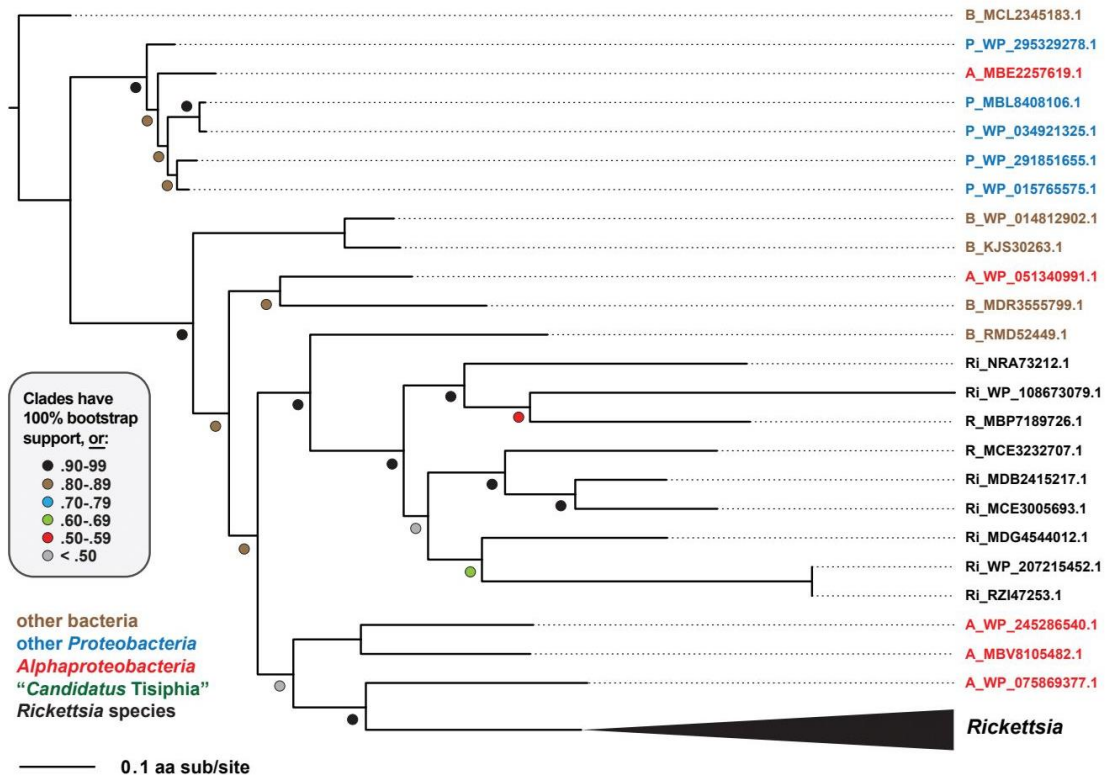


Figure 11. Phylogenetic Tree for the evolution of Pta in Rickettsiae. Accession numbers are present at the end of each branch correspond with NCBI GenBank accession numbers. Accession numbers in brown indicate an unrelated bacterium. Blue represents *proteobacteria*, red represents *alphaproteobacteria*, green represents "*Candidatus Tisiphia*" and black represents *Rickettsia* species. Bootstrap key has .90-.99 confidence in black, .80-.89 in brown, .70-.79 in blue, .60-.69 in green, 0.50-0.59 in red, and anything less than 0.50 has light gray.

We estimated the phylogeny of Pta gene to determine the mode of transmission through Rickettsial evolution. *Desulfobulbus* sp. was used to root the tree due to it being the farthest relation from Rickettsiae species with this gene. The evolution of Pta is visualized as it passed down through evolution vertically. (ex. Figure 11)

Similar to its PHB synthesizing counterparts, Pta is conserved vertically through its evolution in TG, BG, and TRG species. Notably, there are SFG species that also have this gene (*R. japonica*, *R. forneri*, and *R. argasii*). Pta transfers acetyl groups, which is a metabolically important function to all bacterium whether or not they synthesize PHB. We would expect to see this gene to have a role in all rickettsial species.

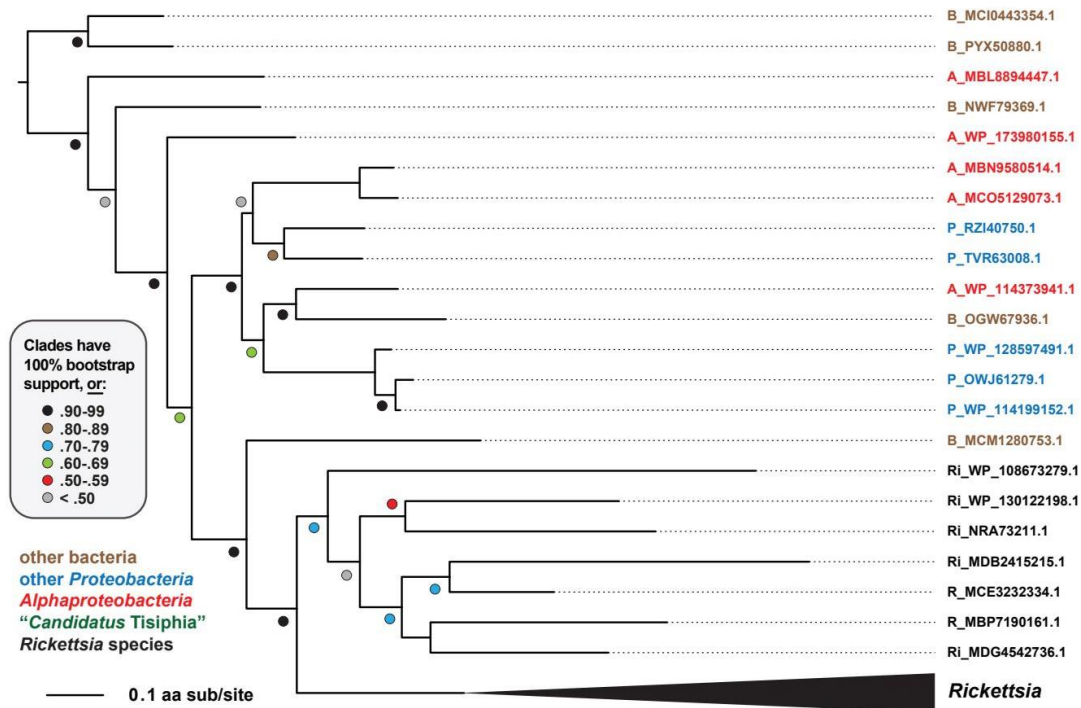


Figure 12. Phylogenetic Tree for the evolution of AckA in Rickettsiae. Accession numbers are present at the end of each branch correspond with NCBI GenBank accession numbers. Accession numbers in brown indicate an unrelated bacterium. Blue represents *proteobacteria*, red represents *alphaproteobacteria*, green represents "*Candidatus Tisiphia*" and black represents *Rickettsia* species. Bootstrap key has .90-.99 confidence in black, .80-.89 in brown, .70-.79 in blue, .60-.69 in green, 0.50-0.59 in red, and anything less than 0.50 has light gray.

We estimated the phylogeny of AckA gene to determine the mode of transmission through Rickettsial evolution. The bacterium to root the tree is of an unknown species. It was chosen due to it being the farthest relation from Rickettsiae species with this gene. The evolution of AckA is visualized as it passed down through evolution vertically. (ex. Figure 12)

Unsurprisingly, AckA is fully conserved in TG, BG, and TRG species as it is needed to begin the utilization of acetate acquired from the host cytosol. The presence of AckA in these species fully outlines the begin-to-end cycle of stealing acetate and transforming it into PHB for long-term storage in nutrient rich environments. Interestingly, this gene is

also vertically conserved in the SFG species *R. philipii*. The presence of this gene was expected to be seen in small amounts similar to Pta due to all bacterium utilizing acetate. The other SFG species must have a more efficient way of utilizing acetate obtained from their host leading to the phasing out of AckA from their genome.

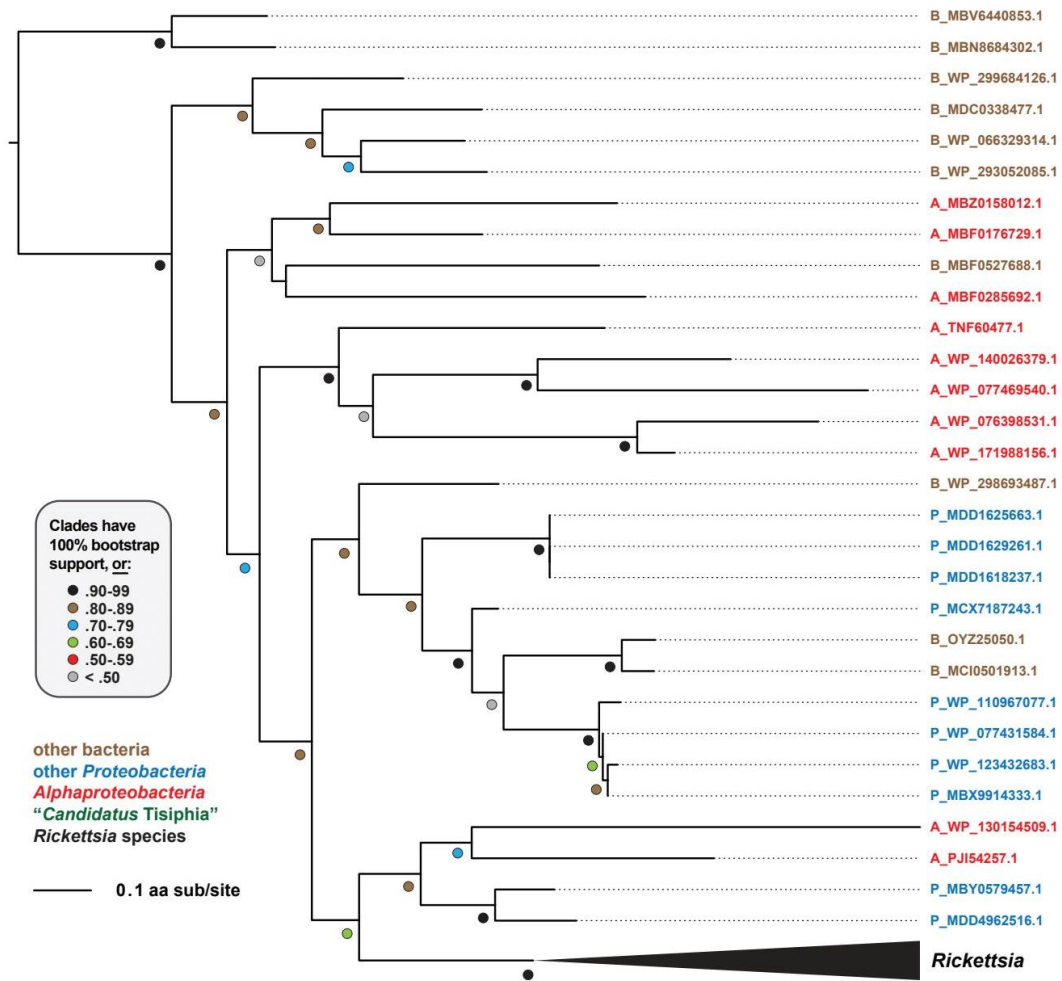


Figure 13. Phylogenetic Tree for the evolution of wecH in Rickettsiae. Accession numbers are present at the end of each branch correspond with NCBI GenBank accession numbers. Accession numbers in brown indicate an unrelated bacterium. Blue represents proteobacteria, red represents alphaproteobacteria, green represents "Candidatus Tisiphia" and black represents Rickettsia species. Bootstrap key has .90-.99 confidence in black, .80-.89 in brown, .70-.79 in blue, .60-.69 in green, 0.50-0.59 in red, and anything less than 0.50 has light gray.

We estimated the phylogeny of WecH gene to determine the mode of transmission through Rickettsial evolution. *Saprospiraceae* was used to root the tree due to it being the farthest relation from Rickettsiae species with this gene. The evolution of wecH is visualized as it was acquired through evolution horizontally from *Gammaproteobacteria*. (ex. Figure 13)

This gene being transferred horizontally also explains why it is not present in BG, TRG, and SFG species. The TG species (*R. typhi* and *R. prowazekii*) picked up this ability to utilize acetate in a novel way for the rickettsia species. The potential O-acetylation of LPS would change the host antibody recognition of TG species.

## **Discussion**

### ***PHB pathway construction:***

According to the RNA sequencing data, genes encoding PhbZ, PhbA, AckA and Pta were all upregulated in *R. typhi* during *C. felis* infection versus HeLa cell infection shown in figure 3. In fleas, a potential reason that PHB would be synthesized is due to the extra metabolites (under the assumption that fleas are not nutrient limiting because they do not suffer negatively from *R. typhi* infection in the laboratory). The overexpression of PHB could also be attributed to differences in their exogenous environments, such as the cat flea being an organism instead of a single cell model or additional stresses not accounted for in a single cell model. This overexpression of PHB genes coincides with what is expected to be upregulated in the hypothesized PHB synthesis pathway as seen in figure 4, highlighted with stars. For example, PhbA serves as a gatekeeper for shunting excess AcCoA into the PHB pathway or acetate metabolism branch, while PhbZ (in conjunction with phasins) regulates the degradation of PHB chains for energy utilization.

Additionally, the upregulation of PhbZ highlights the starvation induced degradation of PHBs that is well characterized for other bacteria like *Azospirillum brasilense*.<sup>19</sup> Once broken down, the PHB molecules can be used in pathways like TCA cycle or beta-oxidation for energy.<sup>37</sup> AckA and Pta are players in the synthesis of acetate in *Rickettsia* species, though acetate import by *R. prowazekii* has been shown (although the mechanism for import remains to be determined).<sup>38</sup>

### ***Species Specific Utilization:***

Not all species of *Rickettsia* are capable of PHB synthesis. It is outlined in figure 5 that only TRG and TG rickettsiae have the genes to synthesize this molecule. There is very little evidence in SFG rickettsiae that these genes are present in their genome, with PhbA being the most present. This would only allow the bacterium to convert Acetyl-CoA to acetoacetyl-CoA which is presumably not metabolically useful. Additionally, some of the SFG rickettsiae have PhbB and PhbC encoded either fully or in a truncated gene sequence. This could potentially mean that these species had the ability to synthesize PHB at one point during their evolution but has since evolved due to no longer needing the carbon storage mechanism, perhaps in the absence of a prolonged extracellular state (e.g., many SFG rickettsiae use secreted effectors Sca2 and/or RickA to polymerize host actin and Sca4 to facilitate intercellular spread).<sup>39</sup> For TRG and TG rickettsiae, the ability to synthesize PHB has seemingly remained a necessity, with full length sequences being present for most analyzed species. This species-specific utilization is also highlighted in photographs taken of rickettsial species using an electron microscope.

### ***Phylogeny:***

Due to the divergence of the ability to synthesize PHB across species of *Rickettsia*, a phylogeny estimation was implemented to track PHB gene presence through their evolutionary process. It was found that the transitional and typhus groups seemed to have retained the ability to synthesize PHB through vertical gene transfer. Thus, the ability to synthesize PHB held some form of evolutionary advantage in the transitional and typhus groups of *Rickettsia* that was seemingly not needed in the spotted fever groups. The vertical transmission makes it harder to say directly where these genes originated since they are conserved in many other rickettsial and alphaproteobacterial lineages. Trees were rooted using the most distant sampled bacteria, which varied across the nine different genes of interest. For example, in figure 6 *Nitrospira* sp. is the bacterium used to root the tree for PhbA. The tree for the evolution of PhbB used *Verrucomicrobiae* as the root. (ex. Figure 7). The root bacterium for PhbC and PhbZ was *Micavibrio*. (ex. Figure 8 and figure 10) Finally the root for PhbCd, *Candidatus Kapabacteria* was the root. (ex. Figure 9). Estimated phylogenies for AckA and Pta showed similar results as the PHB trees, indicating a similar vertical inheritance of these two genes in the *Rickettsia* ancestor. (ex. Figures 11-12) Alternatively, HaloBlast analyses indicated only TG rickettsiae carry the *WecH* gene, which is oddly most similar to analogs from distantly related *Gammaproteobacteria*, particularly certain species of *Pseudomonas aeruginosa*. (Figure 13) *wecH*, annotated as encoding a hypothetical protein, is curiously found in the polysaccharide synthesis operon that is highly variable across *Rickettsia* species and characterized as being critical for O-antigen synthesis in *R. conorii*.<sup>40</sup> Thus, my results indicate that the *Rickettsia* species with the smallest genome size (TG rickettsiae) have the greatest capacity for acetate utilization among all other sampled rickettsiae!

## **Conclusion**

Species of TRG and TG rickettsiae have complete genes responsible for the synthesis of PHB (PhbA, PhbB, PhbC, PhbP), genes for PHB degradation (PhbZ), and genes for acetate degradation into acetyl-CoA (AckA and Pta). These genes were conserved through vertical transfer through the evolution of these groups. The proposed metabolic pathway of PHB synthesis follows the typical PHB synthesis pathway observed in other bacterium capable of this synthesis. The *Rickettsia* utilization of acetate involves acetate degradation to acetyl-CoA for energy use. When in carbon-rich environment, the acetyl-CoA is used in the PHB synthesis pathway resulting in granules visible via electron microscope.

A large drawback to performing this study *in situ* includes having to mine existing data sets. This leads to uncontrolled circumstances such as the RNA sequencing data being a comparison of an organism versus a whole cell. With these drawbacks in mind, future directions would include the movement from *in situ* observations to the wet laboratory environment. PHB presence in both TRG and TG rickettsiae needs to be confirmed. This will be accomplished by using the Nile red detection method in *R. typhi*. Lipid stain Nile red is known to stain the PHB in granules due to its hydrophobic properties.<sup>41</sup> Once stained, the granules will be observed with fluorescence microscopy. Gas chromatography will be used to confirm the presence of PHB in the granules. Other potential avenues of investigation after PHB confirmation will be to explore how the granules are made and what they are comprised of. Arthropods and mammalian cells infected with *Rickettsia* will be observed for granule formation to observe the formation stages and expression of the PHB synthesis genes.

To sum, generated transcripts (unpublished) from *R. typhi* infection assays showed 12 genes significantly upregulated in fleas versus HeLa cells. Of note to my work here, four genes function in two acetyl-CoA metabolic offshoots that are degraded in TIG and SFG rickettsiae. One pathway involves synthesis of PHB, an energy storage molecule found in intracytosolic granules (IG) and utilized by some bacteria during starvation. As IGs are only visible in TG and TRG rickettsiae, this indicates a different, possibly druggable, metabolic strategy in these species versus TIG and SFG rickettsiae. Thus, determining the ability of basal *Rickettsia* lineages and *Tisiphia* species to synthesize PHBs is crucial for understanding if granule formation an important component of vertebrate pathogenesis in TG and TRG rickettsiae (e.g., do granules allow species to persist in arthropod feces before re-infection?). The other acetyl-CoA offshoot involves interconversion of acetate. As *R. prowazekii* can uptake acetate, these rickettsiae likely use host acetate to generate acetyl-CoA. However, TG species also carry a gene capable of further acetate utilization, *wecH*, known to O-acetylate enterobacterial common antigen in *E. coli*. I suspect TG rickettsiae use *WecH* to O-acetylate LPS since *wecH* clusters with other LPS genes. I am excited to initiate experiments that provided the ground work for further dissection of these genomic and metabolic differences using even more robust phylogenomics analyses and experimental investigation.

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