UNDERSTANDING THE ROLE OF *COXIELLA* OUTER MEMBRANE PROTEIN-1 IN RELATION TO THE TYPE IVB SECRETION SYSTEM OF *COXIELLA BURNETII*

A Dissertation

presented to

the Faculty of the Graduate School

at the University of Missouri-Columbia

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

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July 2022

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UNDERSTANDING THE ROLE OF *COXIELLA* OUTER MEMBRANE PROTEIN-1 IN RELATION TO THE TYPE IVB SECRETION SYSTEM OF *COXIELLA BURNETII*

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DEDICATION

I dedicate this dissertation to my father, Robert, whose pride in sharing with strangers that his son is getting his Ph.D. in Microbiology has kept me going; to my mother, Mary Lou, for her patience, support, and love on this journey; to my stepfather Tom, for opening up my world to the possibility of pursuing a Ph.D. and being one to commiserate on the challenges and courteous enough to refrain from asking questions about my progress; to my darling stepmother, Spike, for being a steadfast candle lit in the darkness to ensure I did not lose my way.

To my siblings that have paved the way ahead of me since elementary school and giving their teachers good examples of the high standard of excellence to be expected from the Olivarez kids, I am eternally grateful.

To those near and dear who have transitioned beyond this plane of existence before this chapter in my life was complete, your voices have and continue to guide me and protect me.

To my committee, whose astonishing support and encouragement, despite all the unexpected bumps in the road, have made this possible, many thanks.

ACKNOWLEDGMENTS

Firstly, a profound offering of thanks to my parents, whose unwavering encouragement, belief in my abilities, and support of my career pursuits has allowed me to believe this was something I could achieve. A special thanks to my little sister, Pamela, for her support throughout the various chapters of my graduate school and professional careers. And for their patience and encouragement as I dropped off the face of the earth, a special thanks to my siblings Zazu, B.G., Heuro, Karyn, the Bowers clan and company. To my aunts, uncles, and cousins for their patience and understanding of my distance from the family while I worked to finish my degree. And to my loved ones and friends that have carried me along the way, offering support and encouragement when I was at my lowest, thank you so much Diana, Emi & Liz, JuJu, Kotsil, Ryunosuke & Hiromi, Jennifer & Joey, Nhu, Emily, Aimee, Alex, Bárbara, Candice, Dana, Dandan, Dennis, Freddy, Gerialisa, Jasmine, Johanna, Jorge, Kristy, LaCassidy, Melissa, Mónica, Patricka, Pei-Fen, Seongmi, Vivian, and Wanda.

A very special thanks to my darling children who have lightened up my grad school world: J.P., Grace, Lu, Jo, Stella, Sophia, James, Robert, Alec, Karen, Thomas, Kaiden, and Kylie.

Thanks to my funding sources that have provided the lion's share of my support: NIH NIGMS IMSD Training Grant, NIH T32 Molecular Biology Training Grant, Gus T. Ridgel Fellowship, and Life Sciences Fellowship. And thanks to funding sources that kept me afloat after all those had run their course.

Thanks to the amazing former and current staff in the Molecular Microbiology and Immunology (MMI) Department: Jana Clark, the beloved MMI department mother of all grad students, Shelly Crawford, Karen Ehlert, Kathy LaMere, Scott Greathouse, Kristina Dennis, and Kristen Clark. I could not have done it without you all.

Thanks to the amazing former and current staff in the Veterinary Pathobiology Department: Darlene Ward, Janet Nicholson, Marie Schlup, and Anne Chegwidden who made it possible for me to continue and finish my degree. And especially Robert Barnhart, who kept the building running and prevented me from getting lost in piles of clutter, spilled the tea, provided hours of movie quote-driven conversations and helped me keep my sanity along the journey.

Thanks to the incomparable Jeffory Reeves and Benji Bockting for facilitating so many of the events I helped organize for ACES and SACNAS and the departmental seminars.

Enormous thanks to my mentors who raised me up so that I could achieve heights well

beyond my reach: Debbie Allen, Dr. Raquel Arouca, Dr. Mick Calcutt, Dr. NaTashua Davis, Morgan Crosby-Young, Dr. Mark Hannink, Dr. Heidari Manijeh, and Dr. jeni hart.

Thanks to the former and current staff at the Regional Biocontainment Laboratory, now the Laboratory for Infectious Disease Research: the Late, Great Traci Newell, Catherine Brooks, Emma Tinker-Fortel, Dr. Paul Anderson, Dr. Ami Patel, and a special thanks to Dr. Travis McCarthy who has been so supportive and understanding.

A special thanks to the MU System Graduate Student Leadership Development program for the leadership training and opportunities to expand my professional development to levels I would not have achieved without being selected for the program.

Lastly, thanks to all those wonderful graduate students and post docs that have made time to give back to their community here through the student organizations of Alternative Career Exploration in the Sciences, the MIZZOU SACNAS Chapter, the MU Graduate Collaborative Group. Each of you has helped shaped me into the person I am today and I am grateful for the countless hours we worked to provide for the campus community. And thanks to the MU Graduate Scholars of Excellence for providing the opportunity and support for service-minded graduate students to make an even bigger impact on their community and on the next generation of scholars.

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LIST OF ABBREVIATIONS

°C.....Degrees Celsius

ACCM.....Acidified citrate cysteine media

ACCM-D.....Acidified citrate cysteine media-defined

AGE.....Agarose gel electrophoresis

Amp.....Ampicillin

AMS.....4'-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid

aTc.....Anhydrotetracycline hydrochloride

ATG5.....Autophagy-related protein 5

ATP.....Adenosine tri-phosphate

bp.....Base pairs

BSL-2.....Biosafety Level-2

BSL-3.....Biosafety Level-3

CCV.....*Coxiella*-containing vacuole

CDC.....Centers for Disease Control and Prevention

CFU.....Colony forming units

CLSI.....Clinical and Laboratory Standards Institute

cm.....Centimeter

CO_2.....Carbon dioxide

CSL.....Commonwealth Serum Laboratories

CXXC.....Cysteine, any amino acid, any amino acid, cysteine

Com1.....*Coxiella* outer membrane protein 1

D-.....Dextrorotatory

DAP.....D-alanyl-D-alanine carboxypeptidase

ddH_2O.....Distilled, deionized water

DMSO.....Dimethyl sulfoxide

DNA.....Deoxyribonucleic acid

dot.....Defective organelle trafficking

Dsb.....Disulfide bond

DTT.....Dithiothreitol

EDTA.....Ethylenediaminetetraacetic acid

ELISA.....Enzyme-linked immunosorbet assay

EtBr.....Ethidium bromide

$FeSO_4$ $(H_20)_7$.....Iron sulfate hepta hydrate

FBS.....Fetal bovine serum

g.....Gravitational force

GE.....Genomic equivalent

icm.....Intracellular multiplication

IPTG.....Isopropyl β-D-1-thiogalactopyranoside

Kan.....Kanamycin

kb.....Kilabases

kDa.....KilaDaltons

KPO_4.....Potassium phosphate

kV.....Kilavolts

L-.....Levorotatory

L.....Liters

LB.....Lysogeny broth

LPS.....Lipopolysaccharides

LR White.....London Resin White

μF.....Microfarads

μL.....Microliters

μM.....Micromolar

MAP1LC3a.....Microtubule-associated proteins 1A/1B light chain 3A

Mb.....Megabases

Mβ-CD.....Methyl-β-cyclodextrin

mg.....Milligrams

MIC.....Minimum inhibitory concentration

mL.....Milliliters

mm.....Millimeters

mM.....Millimolar

MW.....Molecular weight

MWCO.....Molecular weight cut-off

N_2.....Diatomic nitrogen

NaCl.....Sodium chloride

ng.....Nanograms

Ni-NTA.....Nickel-nitriloacetic acid

nm.....Nanometers

NMI.....Nine Mile I

NMII.....Nine Mile II

O_2.....Diatomic oxygen

O-LPS.....O-antigen Lipopolysaccharides

OD.....Optical density

P2rx7.....Purinergic receptor P2X, ligand-gated ion channel, 7

PAGE.....Polyacrylamide gel electrophoresis

pBR.....Plasmid, Bolivar & Rodriguez

PBS.....Phosphate buffered saline

pH.....Potential of hydrogen

pPDI.....Plasmid, protein disulfide isomerase

ProA.....Proline synthesis protein glutamate-5-semialdehyde dehydrogenase (A)

ProB.....Proline synthesis protein glutamate 5-kinase (B)

PV.....Parasitophorous vacuole

Q Fever.....Query Fever

RAB5A.....Ras-associated binding protein 5A

RAB7A.....Ras-associated binding protein 7A

RNA.....Ribonucleic acid

RPM.....Rotations per minute

RPMI.....Roswell Park Memorial Institute

SDS.....Sodium dodecyl sulfate

SLIC.....Sequence- and ligation-independent cloning

T4ASS.....Type IVa secretion system

T4BSS.....Type IVb secretion system

TAE.....Trisaminomethane, acetic acid, EDTA

Tris.....Trisaminomethane

TSE.....Trisaminomethane, sucrose, EDTA

V.....Volts

vol/vol.....volume/volume

W.....Watts

wt/vol.....weight/volume

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ABSTRACT

Obligate intracellular bacterial pathogens, like *Coxiella burnetii*, the causative agent of Q Fever, rely upon the host for metabolites and carbon sources for energy and biosynthesis of nucleic acids, proteins, and energy rich molecules necessary for active vegetative growth in the host. Deficiencies in biosynthetic pathways were previously identified through genomic analyses of *C. burnetii*, but bacterial factors contributing to pathogenesis, with the exception of the O-lipopolysaccharide (O-LPS) and the Type IVb Secretion System (T4BSS), remain elusive. The poor efficacy of treatment and vaccine options necessitates understanding how bacterial factors contribute to disease severity, persistence of infections, and inconsistent treatment outcomes. Disulfide bond (Dsb) proteins are integral in the formation and isomerization of disulfide bonds in the T4bSS. Dsb proteins in other bacterial pathogens act upon known virulence factors that promote pathogenicity. The purpose of this study was to characterize the *Coxiella* outer membrane protein 1 (Com1), a putative Dsb protein, establish that it is a functional Dsb protein, and that it is linked to known virulence factors. This work will deepen the understanding in the *Coxiella* field of factors that might serve as alternative targets for therapeutics.

Chapter 1

Introduction to *Coxiella burnetii* and the Current State of the Q Fever Field As it Relates to Disulfide Bond Proteins and Antibiotic Susceptibility

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Coxiella burnetii: Causative Agent of Q Fever

Pathogens found ubiquitously in the environment that cannot be identified using standard culture methods pose considerable health risks to the immune compromised. Such globally distributed pathogens are easily aerosolized, highly infectious in both humans and livestock, and difficult to resolve once the infection has established. The obligate intracellular bacterial pathogen, *Coxiella burnetii* (*C. burnetii*), causative agent of Q fever, is a clear example of a pathogen that is difficult to culture and it requires considerable advancements to mitigate symptoms and resolve infections more effectively (Derrick, 1937; Davis *et al.*, 1938; Harris *et al.*, 2000). *C. burnetii* was developed as a biological weapon during the second World War for its ease of dissemination by aerosol routes, highly infectious nature, and pathology ranging from mild to lethal. These features have also led this pathogen to be categorized as a select agent, necessitating Biosafety Level-3 (BSL-3) precautions to conduct research on virulent strains, making this an important human pathogen to study (Atlas, 2003). The ambiguous febrile symptoms associated with acute Q fever are indistinguishable from common respiratory viral infections and can be easily misdiagnosed since *C. burnetii* requires specialized incubators not typically found in diagnostic laboratories and culture media that is not commercially available. These exacting culture requirements can lead to culture-negative blood test results and patients can be treated for viral infections, instead of being treated with antibiotics for a bacterial infection (Tissot-Dupont *et al.*, 2004; Angelakis and Raoult, 2010). Failure to properly treat the infections can result in the progression to chronic infections, resulting in hepatitis and

myocarditis that lead to potentially lethal outcomes for those infected (Raoult *et al.*, 1990b; Maurin and Raoult, 1999; Raoult *et al.*, 2005). Infected livestock have increased abortions, produce contaminated products, often require culling of infected livestock, and in addition, provide risk for exposure of workers in direct contact with infected fluids. The economic burden of infected livestock makes this bacterial pathogen a global concern for suburban and agricultural communities (Tissot-Dupont *et al.*, 2004; van der Hoek *et al.*, 2012).

C. burnetii has two distinct morphological states, a vegetative and metabolically active large cell variant (LCV) that is observed while the bacteria is in a host cell and a dormant and metabolically inert small cell variant (SCV) that arises under nutrient limiting conditions (McCaul and Williams, 1981; Sandoz *et al.*, 2016). The dormant nature of *C. burnetii* SCV secreted from infected livestock allows the pathogen to persist in soils where the dormant SCVs can be carried on winds to communities downwind of agricultural sites (Schimmer *et al.*, 2010).

This unique Gram-negative, obligate intracellular bacterial pathogen, *C. burnetii*, taxonomically resides in the phylum Pseudomondota, in the order Legionellales, in the family Coxiellaceae, and is currently the only species of the genus *Coxiella*. The closest related bacterial human pathogen to *C. burnetii* is *Legionella pneumophila* (*L. pneumophila*) which, like *C. burnetii*, has a broad host range, and utilizes a Type IVb Secretion System (T4BSS) complex for intracellular growth by transferring bacterial effectors from the bacterial cytoplasm into the cytosol of the host to manipulate the

host into providing metabolic resources for bacterial propagation and protection from the host (van Schaik *et al.*, 2013). One of the unique properties of the *C. burnetii* life cycle that sets it apart from other obligate intracellular bacterial pathogens is that the bacterium does not inhibit or escape from the phagolysosome after being endocytosed by a macrophage (van Schaik *et al.*, 2013). The unique physical properties of the bacterium allow it to thrive in the acidic vacuole, where other bacterial species would be degraded. In fact, this highly acidic environment is crucial to the vegetative replication of *C. burnetii*, allowing for the establishment of a proton gradient between the acidic vacuole and the internal neutral pH of the bacterium, which appears essential to facilitate transport of metabolites from the host into the bacterium, as well as to generate adenosine tri-phosphate (ATP) (Hackstadt and Williams, 1983). Despite the small genome size, approximately 2 megabases (Mb), *C. burnetii* contains a large repertoire of gene products that enable it to proliferate as it transforms the phagolysosome into a parasitophorous vacuole (PV). This vacuole formation does not inhibit the development of the highly acidic environment, but evidence indicates that the host synthesis of reactive oxygen species (ROS) I inhibited which would normally degrade bacteria in the phagolysosome (Akporiaye *et al.*, 1990; Heinzen *et al.*, 1996; Siemsen *et al.*, 2009; Hill and Samuel, 2010; Howe *et al.*, 2010). The most crucial component of the survival of *C. burnetii* in the acidic *Coxiella*-containing vacuole (CCV) is the macromolecular syringe-like T4BSS complex that is predicted to span the bacterial inner and outer membranes, with a long needle-like channel that spans the lipid bilayer of the CCV. Based upon studies of the transfer of bacterial effectors into host cells, the T4BSS allows for the passage of bacterial effectors from the bacterium

into the host (McCaul and Williams, 1981; Chen *et al.*, 2010; Beare *et al.*, 2011).

Known virulence factors of Q fever

Currently, the only known virulence factors for Q fever are the O-antigen lipopolysaccharide (O-LPS) and the T4BSS, the macromolecular syringe complex that transports bacterial effectors from the bacterial cytoplasm of *C. burnetii* across the internal host lipid bilayer of the CCV into the host cell's cytosol (Beare *et al.*, 2011; Carey *et al.*, 2011; Newton *et al.*, 2011; Newton and Roy, 2013). The effectors ultimately allow the internalized bacteria to redirect host cell processes, enabling propagation of *C. burnetii* in the acidic CCV as well as manipulate the host signaling pathways to prevent cytokines and cell surface markers from interacting with components of the immune system that would signal the presence of foreign bacteria inside the infected cells (Luhrmann *et al.*, 2010; Klingenback *et al.*, 2013). There is growing literature focused on identifying and understanding the nature and role of the bacterial effectors that *C. burnetii* secretes into the host cytoplasm of infected host cells in hopes that the interactions between host proteins and the effector molecules can be disrupted, thereby permitting the host proteins to function without the influence of the effectors. However, there are considerable hurdles to targeting effector interactions with host proteins since *C. burnetii* has an impressively broad host range and bioinformatic analyses of the putative effector proteins suggest that there are multiple variants of effectors that could act in a redundant manner (Beare *et al.*, 2009). The broad host range would suggest single therapeutic inhibitors would have to be designed to target several

different proteins or that multiple inhibitors would be needed to disrupt all the variants. The search for a conserved and essential therapeutic target will likely not be found within the more than 300 predicted effectors given the broad host range of the pathogen and the wide variety of targets they must act upon (Chen *et al.*, 2010). The impacts of the acidic pH of the CCV on therapeutic treatments is poorly understood. *C. burnetii* is highly adapted to its unique acidic niche of the CCV, the properties of which make resolution of the infection using standard antibiotics challenging. The acidic environment reduces the efficacy of the doxycycline antibiotics utilized to treat Q fever patients (Raoult *et al.*, 1990a).

Despite nearly 80 years of research on this important agricultural and human pathogen, little is known about how it causes disease in animal or human hosts compared to other pathogens. Progress has largely been slowed until recently by an inability to culture this microbe outside of host cells. Recent developments have enabled sustained growth of *C. burnetii* outside of the host cell using modified, acidified citrate cysteine medium (ACCM-2) that has also led to the advent of genetic tools, facilitating the creation of mutants to elucidate the molecular mechanisms behind the well-known phenotypes of this pathogen (Osmand *et al.*, 2011; Beare *et al.*, 2008). However, the prolific regulatory guidelines for working on select agents and higher costs associated with BSL-3 facilities, has led to all current genetic studies focused on the avirulent strain, RSA 439 Nine Mile phase II (NMII), that lacks twenty open reading frames (ORFs) predicted to be involved in lipid biosynthesis (Stoker and Fiset, 1956; Hoover *et al.*, 2002). The severe truncation in the LPS in NMII led to the discovery that LPS is a virulence factor in Q

fever (Kazár *et al.*, 1974; Moos and Hackstadt, 1987; Beare *et al.*, 2018). It has been inferred that the T4BSS is an additional virulence factor, since the bacteria lacking this syringe complex are unable to replicate intracellularly. Thus, the polarly localized T4BSS is an essential factor for pathogenicity of Q fever (Voth *et al.*, 2009; Morgan *et al.*, 2010; Beare *et al.*, 2011). The inability of strain NMII to induce disease prevents the mechanisms of pathogenesis from being understood in context of disease in an immune competent host. Multiple proteomic studies of *C. burnetii* have found that when cultured intracellularly or in axenic media, components of the T4BSS are present in both conditions (Sandoz *et al.*, 2014; Warrier *et al.*, 2014; Dresler *et al.*, 2019; Moormeier *et al.*, 2019). These findings are valuable since a better understanding of how the individual protein components are formed that comprise the T4BSS requires culturing *C. burnetii* outside the host cell environment. Apart from the TB4SS and O-LPS, no other bacterial virulence factors have been validated experimentally. For these reasons, a better understanding of the molecular mechanisms of pathogenesis are required to identify novel therapeutic targets to facilitate clearance of chronic infections.

In the search for virulence factors, a 27 kDa immunodominant protein was identified from *C. burnetii*-infected Buffalo Green Monkey kidney cells, mice, guinea pigs, rabbits, and humans (Müller *et al.*, 1987; Schmeer, 1988; Hendrix *et al.*, 1991; Sekeyová *et al.*, 2008). The 27 kDa *Coxiella* outer membrane protein 1 (Com1) was ultimately characterized from rabbits, (Hendrix *et al.*, 1990). Analysis of the DNA sequence encoding Com1 revealed that the gene encoded a protein with a CXXC amino acid motif characteristic of the catalytic site of protein disulfide oxidoreductases, the

hallmark feature of disulfide bond (Dsb) proteins (Hendrix *et al.*, 1993). An in-depth analysis of *com1* sequences from several isolates of *C. burnetii* identified that strains associated with endocarditis and hepatitis shared sequence identity at several key residues, suggesting a link between *com1* sequence and virulence (Zhang *et al.*, 1997). All attempts to isolate transposon knockout mutants of com1 by several investigators have failed (personal communication from Dr. Paul Beare), suggesting that Com1 may be essential for both extracellular and intracellular growth of *C. burnetii*.

The lack of knowledge concerning bacterial factors and their roles during infection of hosts have prevented advancements in treatment of humans afflicted with Q fever over the past three decades since the current treatment regimen of patients with doxycycline and hydroxychloroquine was established (Raoult *et al.*, 1990a). Despite the widespread use of this treatment modality, its poor effectiveness requires patients afflicted with acute infections to take antibiotics for up to two weeks to resolve the infection, while patients with chronic Q fever can be placed on antibiotics for up to 4 years, though the resolution of the infection is not a certainty even after prolonged antibiotic therapy (Angelakis and Raoult, 2010). It is well known that *C. burnetii* is highly resistant to environmental stressors found in soils in addition to common methods used for inactivating other bacteria, such as ultraviolet light, detergents, and surfactants (Babudieri, 1959; McCaul *et al.*, 1981). However, there is a glaring gap in the current literature that is unable to determine if and to what degree the natural stability and persistence of the metabolically dormant SCV of *C. burnetii* contributes to the poor resolution of infections upon treatment with prolonged antibiotic therapies.

Determining the nature of persistence and antibiotic susceptibility has been hampered for nearly 70 years since there were no methods available for the successful axenic culturing of *C. burnetii* until the recent development of the axenic culture media ACCM and its progressively improved formulations in the past ten years. The studies prior to the development of axenic culture media have been based on the biochemical properties of the CCV, which have led to minor, but improved treatment of patients afflicted with Q fever, principally, the addition of hydroxychloroquine in conjunction with doxycycline antibiotic therapy (Raoult *et al.*, 1990a). As it has already been mentioned, the harsh acidic environment of the CCV is a barrier to delivering effective therapeutics to resolve *C. burnetii* infections. Despite lacking axenic culturing techniques, elegant studies were able to conclude nearly 30 years ago that alkalinizing the CCV with the use of lysosomotropic agents, like hydroxychloroquine, shifts the pH of the CCV closer to neutral, which results in the clearance of the infection in cell lines (Raoult *et al.*, 1990a). Despite improving the efficacy of current antibiotic therapies in cell culture, clinical improvements have proved less impressive with patients subjected to potentially years of antibiotic treatment that may not resolve the infections. Based on the successful development of several vaccines against bacteria, there are groups that focus on exploring the efficacy and safety of a prophylactic strategy to protect livestock, agricultural workers, military personnel, and communities surrounding agriculture areas from exposure to aerosols shed from infected livestock in urine, feces, milk, and birthing products (Atlas, 2003; Tissot-Dupont *et al.*, 2004). However, current vaccine strategies pose high risks for those with previous exposure to this environmentally ubiquitous bacterial pathogen. Since an estimated 60% of those that

have been infected with *C. burnetii* are asymptomatic, there is a high likelihood that previous exposure can result in severe adverse side effects at the site of immunization and systemically, including abscesses that require surgical removal and granulomas that would negate the benefits of the vaccination without thorough screening of those receiving vaccinations to determine any existing exposure to *C. burnetii* (Smadel *et al.*, 1947; Benenson and Tigertt, 1956; Lackman *et al.*, 1962; Bell *et al.*, 1964; Kazár *et al.*, 1982; Marmion *et al.*, 1984; CSL, 2009). The exact mechanisms underlying this hypersensitivity have yet to be determined. Therefore, a need remains to find better therapeutic targets for the treatment and prevention of *C. burnetii* infections in humans. It is already known that the T4BSS complex is essential for intracellular growth of *C. burnetii* and without it, no infections are established and no pathology can develop. Thus, there is an intriguing appeal to explore how inhibition of T4BSS function or formation could impact ongoing infections as well as potential synergistic effects of combining this approach with current antibiotic therapies.

Disulfide bond proteins and their role in virulence

A growing literature has consistently shown that Dsb proteins are key contributors to virulence factors in an ever-expanding list of bacterial pathogens like *Burkholderia cepacia*, *Burkholderia pseudomallei*, *Corynebacterium diphtheriae*, *Francisella tulerensis*, and *Mycobacterium tuberculosis* (Hayashi *et al.*, 2000; Qin *et al.*, 2008; Premkumar *et al.*, 2013; Ireland *et al.*, 2014; Ren *et al.*, 2014; Reardon-Robinson *et al.*, 2015). The highly conserved active sites of Dsb proteins and the critical role they have in ensuring

virulence factors (including components of the T4BSS complex) are properly folded and biologically active make them an attractive target for further research into how they can be utilized as therapeutic alternatives to antibiotics. Targeting of Dsb protein activity would be especially beneficial for Q fever since the survival of the pathogen intracellularly is entirely reliant on a functioning T4BSS (Beare *et al.*, 2011). Bioinformatic analysis of the amino acid sequences of the *C. burnetii* protein components that comprise the T4SS complex reveals that of all 25 T4SS protein components, 13 are putative substrates of Dsb proteins since they contain 2 or more cysteine residues (Table 1-1). The main component of the core complex that spans the periplasm is IcmE/dotG (CBU_1627) which has 15 cysteine residues of 1039 residues in total. Of the 13 putative Dsb protein substrates, 7 are predicted to have a transmembrane domain or be either anchored in the inner or outer membrane, making them likely substrates of a periplasmic Dsb protein (Segal *et al.*, 2005). Given the essential function of the *C. burnetii* T4BSS, the value of identifying what proteins are involved in the proper folding of the components of this virulence factor is immense.

Role of Dsb proteins in Gram negative bacteria

Unlike the well-studied bacterial pathogen, *E. coli*, that can replicate in a wide array of environments and propagate using a variety of metabolic and aerobic pathways, obligate intracellular bacteria often have reduced genomes since they rely upon their host environment to provide many of the essential nutrients and metabolites required for replication. *E. coli* strains can encode nearly a dozen distinct Dsb proteins, which

Table 1-1. List of *C. burnetii* NMI T4BSS proteins with predicted localization and cysteine residue counts.

C. burnetii NMI T4BSS Protein Description	Locus Tag	Accession	Predicted Localization	Cysteines
IcmH/DotU	CBU_0321		Inner membrane (IM)	3
IcmF	CBU_0322		IM	6
IcmB/DotO	CBU_1622		IM/Cytoplasm (C)	6
IcmJ/DotN	CBU_1623		C	6
IcmD/DotP	CBU_1624		IM	0
IcmC/DotE	CBU_1625		IM	2
IcmG/DotF	CBU_1626		IM/Periplasm (P)	0
IcmE/DotG	CBU_1627		IM/P	15
IcmK/DotH	CBU_1628		Outer membrane (OM)	1
IcmL.1/DotI.1	CBU_1629		IM	1
IcmL.2/DotI.2	CBU_1630		IM	0
IcmN/DotK	CBU_1631		OM/P	2
IcmO/DotL	CBU_1632		IM/C	4
IcmP/DotM	CBU_1633		IM/C	1
IcmQ	CBU_1634		C	3
CoxigA	CBU_1634a		Unknown	0
IcmT	CBU_1641		IM	0
IcmS	CBU_1642		C	2
DotD	CBU_1643		OM/P	1
DotC	CBU_1644		OM/P	1
DotB	CBU_1645		C	3
DotA	CBU_1648		IM	7
IcmV	CBU_1649		IM	5
IcmW	CBU_1650		C	1
IcmW	CBU_1652		P	0

can be capable of functioning redundantly in the event one or more of the other Dsb proteins are functionally impaired. However, the genome of *C. burnetii* is less than half the size of the most commonly researched *E. coli* strains and bioinformatic analysis of the genomes of over a dozen *C. burnetii* strains reveals only four types of Dsb proteins present in each of the strains. Each of the four Dsb protein types has distinctly different properties and functions. Though sequence annotation varies considerably among the strains of *C. burnetii*, each strain to date has only four Dsb proteins that can be separated into four classes: DsbA, DsbB, DsbA-like, and DsbD proteins (Kadokura *et al.*, 2003). This lack of redundancy is particularly noteworthy since inhibiting a Dsb protein in a pathogen that possesses multiple redundant homologs would make for a poor therapeutic target. The presence of four unique Dsb proteins could potentially offer multiple and independent therapeutic targets to consider for future therapeutic development. Additional details about the well-studied structure and functions of the above classes of Dsb proteins is required to appreciate the rationale for pursuing this line of research.

Function of Dsb proteins

Dsb proteins, in addition to having highly conserved active site motifs, have very well-defined biochemical functions based upon previous work done in the *E. coli* system (Bardwell *et al.* 1991; Akiyama *et al.*, 1992; Kamitani *et al.*, 1992; Bardwell *et al.*, 1993). These studies have determined the bacterial periplasm is where DsbA and DsbA-like proteins localize that are directly involved in disulfide bond formation on linear

peptides transported across the inner membrane into the periplasm. These DsbA proteins exist in an oxidized state with the cysteines of their active site covalently bound in a disulfide bond.

The formation of a disulfide bond is a catalytic reaction that occurs when a Dsb protein with cysteine residues joined by a disulfide bond (S-S) encounter a substrate protein with cysteines in a reduced state (S-H). When encountering a peptide substrate, a nucleophilic attack from the first cysteine of the active site on the cysteine residue of the substrate covalently binds DsbA with the substrate (Figure 1-1). Then a nucleophilic attack from the second cysteine of the enzyme active site allows the complete formation of a disulfide bond on the peptide substrate, leading to the reduction of DsbA (Kadokura *et al.*, 2004). In a reduced state, DsbA is unable to oxidize peptide substrates. The regeneration of the DsbA oxidative state from a reduced state occurs through interactions with an inner membrane bound Dsb protein known as DsbB, which is essential for the regeneration of the oxidation state of DsbA. Regeneration of the oxidative state of DsbA results in the reduction of DsbB; however, the electron transport chain, which includes quinones as electron transporters, is able to oxidize DsbB (Raina and Missiakas, 1997). Together these protein pairs form the Dsb oxidative pathway that allows peptides transported across the inner membrane into the periplasm to have disulfide bonds formed between the cysteine residues. Both DsbA and DsbB in *E. coli* systems exist as monomers in the periplasm and inner membrane, respectively (Landeta *et al.*, 2018). Of note, when *Coxiella* outer membrane protein 1 (Com1) was initially identified, based on sequence availability at the time, it was found to be most

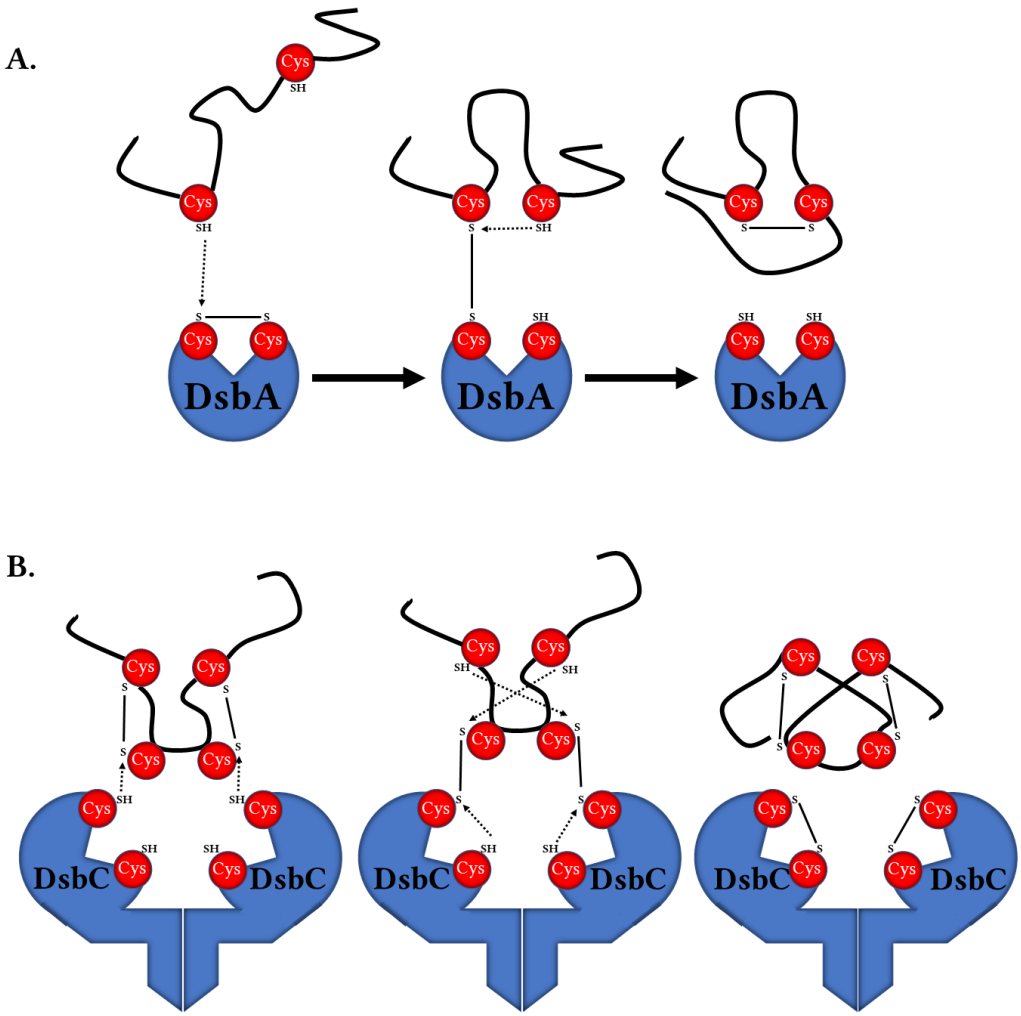

Figure 1-1. Formation and isomerization of disulfide bonds with DsbA and DsbC. Panel A: Depiction of typical nucleophilic attacks between cysteine residues of substrate and DsbA to generate disulfide bonds in substrate proteins. Panel B: Depiction of typical nucleophilic attacks between cysteine residues of substrate and DsbC to isomerize disulfide bonds in substrate proteins.

similar to DsbA proteins, which had been identified in a publication a few years prior (Bardwell *et al.*, 1991; Hendrix *et al.*, 1993). However, with the expansion of protein databases over the following decades, a more homologous Dsb protein has been found to be more characteristic of the biochemical properties exhibited by Com1. Also, a DsbA-like protein of the closely related *L. pneumophila*, DsbA-2, was initially mistaken as a monomer until the expansion of protein databases allowed additional insight to identify key sequences, which are characteristic of binding domains found in homodimer Dsb proteins elaborated upon below (Jameson-Lee *et al.*, 2011; Kpadeh *et al.*, 2013; Kpadeh *et al.*, 2015). There is currently no published information on the roles of the *C. burnetii* DsbA, DsbB, DsbA-like, and DsbD proteins. What is clear from bioinformatic analysis of DsbA and DsbB is that they lack any sequences that would suggest the presence of a binding domain characteristic of homodimers, are transcribed as an operon, and that they are predicted to localize in the periplasm and anchored in the inner membrane, respectively.

When periplasmic proteins contain more than two cysteine residues, there is a probability that the initial disulfide bond formed between two of the cysteines may not result in a functional structure (Missiakas *et al.*, 1994; Shevchick *et al.*, 1994). In this scenario, a proof-reading mechanism is necessary to break the initial disulfide bond(s) and rearrange the disulfide bond with the other cysteine residue(s) so that the correct functional structure can be formed (Zapun *et al.*, 1995). This rearrangement of disulfide bonds by Dsb proteins is known as the Dsb isomerization pathway. This pathway in many respects is distinct from the Dsb oxidative pathway. Principally, the Dsb proteins

involved in the Dsb isomerization pathway function predominantly as homodimers or have multiple domains with CXXC motifs. The oxidative states of the players are inverted as well, since the cysteines of the substrates with incorrectly formed disulfide bonds perform a nucleophilic attack on the reduced active sites of the homodimer DsbC, the isomerase localized in the periplasm and Dsb proof-reader. DsbC is then reduced by a large monomeric inner membrane bound DsbD protein, allowing for DsbC to be continually active to correct misfolded proteins. Early studies in *E. coli* determined that the homodimer DsbC has distinctly different kinetics of how it can make or break disulfide bonds when compared to DsbA proteins. Additionally, the enzyme kinetics of DsbC can be altered to mirror kinetics of DsbA by changing critical amino acids necessary for binding the two homodimers of DsbC together (Bader *et al.*, 2001). The alteration of DsbC enzyme kinetics by inhibiting homodimer formation suggests that as a monomer, DsbC, functions no differently than DsbA in the *E. coli* model, which was verified experimentally in Δ*dsbA* mutants being rescued by monomeric variants of DsbC (Bader *et al.*, 2001). As previously mentioned, there is a homolog for DsbD in *C. burnetii*, but no annotations for DsbC proteins. Closer bioinformatic analysis of the DsbA-like protein, Com1, reveals that unlike DsbA, it possesses amino acid sequence domains characteristic of other homodimeric DsbC proteins, suggesting that it could function as a Dsb isomerase protein. Unlike the *C. burnetii dsbA* and *dsbB* genes that are found on a single operon throughout the different strains of *C. burnetii*, *Com1* and *dsbD* genes have no discernible connections in genomic placement. Like the two Dsb proteins putatively involved in the Dsb oxidation pathway, Com1 and DsbD have little published information regarding their function. However, Com1 and DsbD are predicted to

localize in the periplasm and inner membrane, respectively. There have been speculations about the function and association of Com1 with the severity of Q fever because of sequence similarities in Com1 proteins found among strains isolated from chronic infections compared to Com1 sequences among strains isolated from acute Q fever infections in humans (Zhang *et al.*, 1997). It should be noted that the nucleotide sequences of *com1* genes are so uniquely associated with *C. burnetii* that primers targeting the *com1* gene were developed early on as a method to quantify the genomic copies of the bacterium since only one copy of *com1* was present in the genome (Brennan and Samuel, 2003). The *com1* gene has also been used as an alternative diagnostic method to blood culture testing to ascertain if a patient was colonized with *C. burnetii* (Zhang *et al.*, 2003). With the advent of more sequenced *C. burnetii* genomes and the emergence of multispacer sequence typing of intergenic regions, more informative sites within the genomes were selected that enabled researchers to use 10 different spacers to analyze 159 strains of *C. burnetii*, resulting in 30 different genotypes that showed clustering among the strains associated with disease severity of the infections (Glazunova *et al.*, 2005). However, disease severity cannot be entirely attributed to the bacterial virulence factors. Studies support that a significant factor in disease progression and severity is linked to the immune compromised state in humans (Maurin and Raoult, 1999; van der Hoek *et al.*, 2011). Stemming from initial reports of the Netherlands Q fever outbreak of 2007-2010, a recent study of the victims has established an association between variations of human innate immune proteins RAB7A, P2RX7, MAP1LC3A, and ATG5 (involved in phagosome maturation, microbial clearance, and two involved in autophagy, respectively). An association between

variations in those proteins and the development of chronic Q fever was found (Wouda and Dercksen, 2007; Enserink, 2010; van den Wijngaard *et al.*, 2011; Jansen *et al.*, 2019). Specifically, these studies found that variations in RAB5A, P2RX7, MAP1LC3A, and ATG5 were associated with protection against chronic Q fever development (Jansen *et al.*, 2019). Considerably more work is necessary to understand the complex host-pathogen interactions that determine severity of Q fever.

A novel approach to exploring factors involved in Q fever pathogenesis

Alternative approaches are need to improve treatment options for Q fever. One such approach is to understand the role of Dsb proteins in Q fever pathogenesis. Novel therapeutic targets for this and other difficult to treat bacterial pathogens have the potential to emerge as well from this research direction. Previous immunological studies of *C. burnetii* in various animal models (Green Monkey Kidney cells, guinea pigs, and rabbits) identified an unknown 27 kDa protein in Western blots (Müller *et al.*, 1987; Schmeer, 1988). Subsequent studies of a 27 kDa immunodominant protein successfully cloned and sequenced the gene encoding the 27 kDa protein which was named *Coxiella* outer membrane protein 1 (Com1). Although, there is no certainty that the 27 kDa proteins identified previously by Western blot were Com1, the 27 kDa Com1 protein was found to have the hallmark CXXC motif in the amino acid sequence, indicative of the active site of Dsb proteins (Hendrix *et al.*, 1990; Hendrix *et al.*, 1993). This discovery lead the initial investigators to logically suggest that Com1 may function as a Dsb protein (Hendrix *et al.*, 1993). The immunodominant nature of Com1 inspired

multiple groups to explore the utility of Com1 as a vaccine candidate. However, initial studies found that Com1 generated weak protection for mice infected with strain NMI (Zhang and Samuel, 2003). Efforts in the USA continue to seek out out effective Q fever vaccine candidates that can meet Food and Drug Administration approval.

In the thirty years since the earliest descriptions of the immunodominant protein Com1, important questions remain unanswered regarding its structure, function, and role in Q fever pathogenesis. The purpose of this dissertation is to generate evidence demonstrating biochemically and genetically that Com1 is a Dsb protein and that it interacts with components of the T4BSS complex. In addition, this work will identify currently unknown substrates that can be researched further as potential therapeutic targets for treatment of Q fever. In this manner, the nature of Com1 structure, function, and gain insight into its role in Q fever pathogenesis can be elucidated and lay the groundwork for future studies that can explore how these data can benefit the development of novel therapeutics for the treatment of those afflicted with Q fever and expedite their recovery.

Bioinformatic analysis of putative Dsb protein Com1

Bioinformatic analysis of Com1 is the initial method utilized to examine existing nucleotide sequences and published data sets studying the proteome and transcriptome of *C. burnetii* infections in cell lines that guided the following studies characterizing this poorly understood protein. In depth bioinformatic comparisons of *com1* at the

nucleotide and amino acid level with well-studied Dsb proteins from other Gammaproteobacteria species focused insight into the functional activities of Com1 compared with Dsb proteins with high sequence similarities, which enabled the prediction of critical binding and functional domains. Com1 is the only putative Dsb protein in *C. burnetii* for which the gene has been cloned and sequenced. As yet, there have been no published work on the other three Dsb proteins predicted by sequence homology of functional domains from published genomes of strains. Dsb protein activity is dependent upon the regeneration of periplasmic Dsb protein redox states by the inner membrane bound Dsb proteins, so a detailed understanding of the similarities and differences of the putative Dsb partners should provide valuable clues of how these interactions affect function among strains associated with varying severities of Q fever manifestation. The *C. burnetii* strains currently used for research have been cultured only through cell lines and animal models for the first seven decades of Q fever studies, meaning that no exacting methods were available until the development of ACCM to isolate distinct clonal populations of *C. burnetii* from a bacterial colony on a solid medium. Continuous culturing of polyclonal populations used for infection studies may exhibit small genomic variations. Generation and analysis of the sequences from clonally isolated populations would ascertain the fidelity of the genomic sequences. Cumulatively, these bioinformatic analyses would provide foundational information about the genes and proteins under examination in the subsequent research aims and would ensure the accuracy of structural, biochemical, and substrate results.

Biochemical characterization of Com1

Biochemical characterization of Com1 informed by bioinformatic analyses permits Com1 to be confirmed as a Dsb protein with the insulin disulfide reduction assay (Holmgren, 1979). This insulin disulfide reduction assay can identify the class of Dsb proteins to which Com1 belongs, since the enzymatic kinetics of the Dsb oxidation versus isomerization are distinctly different (Darby *et al.*, 1998). The bioinformatic analysis of published protein sequence data should identify the essential proline residue opposite the Dsb active site (Kadokura *et al.*, 2004). Additionally, the presence of a dimerization domain on Com1 may be confirmed through sequence alignments of well characterized homodimeric DsbC proteins. These simple biochemical assays will establish the key functional nature of Com1 as a Dsb protein and should provide clearer details as to the role of Com1 in the context of the enzymatic kinetic differences between the Dsb oxidation and isomerization pathways. These data will direct the *in vivo* studies that ask if the unique Com1 protein can functionally rescue *dsb* mutants in a library of *E. coli* strains deficient in Dsb activity.

Complementation studies of com1 with E. coli dsbC mutant

Genetic tools and expertise for cloning in *C. burnetii* are still relatively novel for this field of study and cloning can take months before a single mutant can be confirmed and additional months before mutant characterization can begin. For these reasons, utilization of the *dsb* mutant library Dr. Bardwell's group has assembled in *E. coli* to assess the function of *C. burnetii* putative Dsb proteins in rescuing *E. coli dsb* mutant

strains (Ren and Bardwell, 2011). The readout for successful complementation made use of a protein disulfide isomerase reporter plasmid, pPDI. Based on the pBR322 vector, Ren and Bardwell modified the ampicillin resistance cassette by introducing two additional cysteine residues that do not alter β-lactamase activity when a disulfide bond is formed between the two native cysteine residues, but is non-functional if disulfide bonds are formed between a native cysteine and a non-native cysteine residue. Expression vectors carrying *C. burnetii dsb* genes were introduced into the pPDI-containing *E. coli* Δ*dsb* strains and plated onto LB agar plates with high concentrations of ampicillin to assess the ability of *C. burnetii* Dsb proteins to correctly isomerize the disulfide bonds of β-lactamase (Ren and Bardwell, 2011). The pPDI plasmid is therefore a sensitive detector of Dsb isomerase activity with which to test Com1 enzymatic activity. Replacement the *E. coli* antibiotic selection marker used for the *C. burnetii* auxotrophic cloning vector, pJB-ProBA-2xHA-Amp, with a kanamycin resistance marker was required to not interfere with the β-lactamase susceptibility readout of the assay; resulting in the generation of the new auxotrophic vector, pJB-ProBA-2xHA-Kan. The pPDI detector plasmid, combined with the library of *E. coli dsb* mutant strains demonstrated that *C. burnetii* Com1 has protein disulfide isomerase activity, validating it as a Dsb protein and providing sufficiently compelling evidence to proceed with genetic studies in *C. burnetii* to assay the effects of Com1 dysfunction in *C. burnetii.*

Identifying C. burnetii protein substrates of Com1

Effective tools for genetic manipulation of *C. burnetii* have only existed for the past ten years and the first generation of cloning vectors that utilized antibiotic selection markers frequently resulted in spontaneous development of resistance (Sandoz *et al.*, 2016). Development of new cloning and expression vectors that took advantage of the incomplete metabolic pathways for the synthesis of key amino acids allowed for the creation of amino acid-based auxotrophic selection markers for exogenous genetic elements. These amino acid-based auxotrophic cloning tools, when paired with the latest generation of defined ACCM-D culture methods, allowed for stable genetic transformations in *C. burnetii* and virtually eliminated the challenges of spontaneous resistance development associated with antibiotic selection markers (Sandoz *et al.*, 2016). Implementation of this latest generation of auxotrophic genetic tools is a time consuming and labor intensive process demanding specialized instrumentation and flawless techniques to prevent contamination of long term cultures. Careful adherence to published protocols and supportive advice and unpublished tips from Dr. Paul Beare for use of the genetic tools he and his team have developed has enabled the generation of mutants in the nonvirulent *C. burnetii* NMII strain. Identification of the *C. burnetii* substrates of Com1 was determined with a Com1 mutant that is impaired in completing the creation of disulfide bonds in its substrates because it lost the essential proline opposite the active site and remains covalently bonded to protein substrates. This substrate-capturing point mutation was transformed into the NMII strain of *C. burnetii* on an inducible expression vector encoding the 6x-Histidine-tagged Com1 P219T point mutant. Controls included an expression vector encoding a wild-type 6x-Histidine-tagged Com1.

Establishing Com1 as a Dsb protein was determined using 1) bioinformatic analysis of the nucleotide and amino acid sequences of *com1*, 2) biochemical characterization of enzymatic activity of Com1 and Com1 with point mutations 3) and *com1* expressed exogenously in a library of *dsb* mutant strains of *Escherichia coli* (*E. coli*) lacking genes encoding various Dsb proteins, and *in vivo* determination of the redox state of recombinant Com1 expressed in NMII. Identification of the Com1 periplasmic substrates was determined with the recombinant Com1 P219T expressed in NMII and analysis of the purified protein extracts by mass spectrometry.

Chapter 2

Characterizing the Com1 Protein

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Summary

Obligate intracellular bacterial pathogens, like *Coxiella burnetii*, the causative agent of Q fever, are entirely reliant upon the host for metabolites and carbon sources for energy and biosynthesis of nucleic acids, proteins, and energy rich molecules necessary for active vegetative growth in the host. While the deficiencies in biosynthetic pathways have been previously identified through detailed bioinformatic analysis of genomic sequences for *C. burnetii*, the bacterial factors that contribute to pathogenesis, with the exception of the O-lipopolysaccharide (O-LPS) and the Type IVb Secretion System (T4BSS), remain elusive. The poor efficacy of current disease treatment and vaccine options necessitates identifying and understanding how bacterial factors contribute to disease severity, persistence of infections, and inconsistent treatment outcomes. Disulfide bond (Dsb) proteins are integral in the formation and isomerization of disulfide bonds in bacterial macromolecular transmembrane structures like the T4BSS. Dsb proteins in numerous bacterial pathogens have substrates that are known to be virulence factors that promote pathogenicity. The purpose of this study is to characterize the *Coxiella* outer membrane protein 1 (Com1), a putative Dsb protein and establish that it is a functional Dsb protein and that it is linked to known virulence factors. This work will deepen the understanding in the *Coxiella* field of factors that might serve as alternative targets for therapeutics.

Introduction

The globally ubiquitous pathogen, *C. burnetii*, causative agent of Q fever, is easily aerosolized, highly infectious in both humans and livestock, and difficult to resolve

once the infection has established. This pathogen is a select agent, necessitating Biosafety Level-3 precautions to conduct research on virulent strains. The impacts that this bacterial pathogen has had on human health and the agricultural economy makes this an important human pathogen to study (van Schaik *et al.*, 2013). Ambiguous febrile symptoms associated with acute Q fever are indistinguishable from common respiratory viral infections and can be easily misdiagnosed. Advancements are needed to mitigate symptoms and resolve infections more effectively. Identifying *C. burnetii* through culture methods requires specialized equipment not typically found in diagnostic laboratories and culture medium that is not commercially available. The absence of these exacting culture requirements can lead to culture-negative diagnosis and antiviral therapeutic interventions that cannot clear the bacterial infection (Houpikian and Raoult, 2005). Failure to properly treat the infections can result in the progression to chronic infections resulting in hepatitis and myocarditis that can lead to potentially lethal outcomes for those infected (Maurin and Raoult, 1999). The economic burden of infected livestock on the agricultural industry in respect to increased abortions of infected animals, contaminated products, exposure of workers to infected fluids and exposure of communities downwind of contaminated agricultural sites makes this bacterial pathogen a global concern for suburban and agricultural communities (van der Hoek *et al.*, 2012).

Currently, the only known virulence factors for Q fever are O-LPS antigen and the T4BSS, the macromolecular syringe complex that transports bacterial effectors from the bacterial cytoplasm of *C. burnetii* across the internal host lipid bilayer of the *Coxiella*

containing vacuole (CCV) into the cytosol of the host cell (Beare *et al.*, 2011). The bacterial effectors interact with host cell proteins, allowing the internalized bacteria to redirect host cell processes and enabling propagation of *C. burnetii* in the acidic CCV (Beare *et al.*, 2011; Carey *et al.*, 2011; Newton and Roy, 2011; van Schaik *et al.*, 2013). Additionally, the bacterial factors manipulate the host signaling pathways to prevent cytokines and cell surface markers from interacting with components of the immune system that would signal the presence of foreign bacteria inside the infected cells (Voth *et al.*, 2009; Luhrmann *et al.*, 2010). *C. burnetii* is highly adapted to its unique acidic niche of the CCV, the properties of which make resolution of the infection by standard antibiotics challenging. In addition, the acidic environment reduces the efficacy of the doxycycline antibiotic utilized to treat Q fever patients (Raoult *et al.*, 1990a).

The current Q fever treatment regimen of doxycycline and hydroxychloroquine was established over three decades ago (Raoult *et al.*, 1990a). Despite the widespread use of this treatment modality, its poor effectiveness requires patients afflicted with acute infections to take antibiotics for up to two weeks to resolve the infection. In contrast, patients with chronic Q fever can be placed on antibiotics for up to 4 years, though the resolution of the infection is not a certainty even after prolonged antibiotic therapy (Angelakis and Raoult, 2010). Therefore, a need remains to find better therapeutic targets for the treatment and prevention of *C. burnetii* infections in humans. The T4BSS complex is essential for intracellular growth of *C. burnetii*. Without the T4BSS, bacteria are unable to replicate and no pathology can develop. The essential role of the T4BSS warrants further exploration into how inhibiting T4BSS functions or T4BSS formation

could impact ongoing infections as well as potential synergistic effects of combining the impairment of T4BSS function with current antibiotic therapies.

A growing literature consistently finds that disulfide bond (Dsb) proteins are key contributors to virulence factors in an expanding list of bacterial pathogens. When Dsb proteins are deleted in these pathogens, the proteins responsible for virulence become misfolded and inactive, resulting in a loss or severe attenuation of the disease phenotypes. Deletion of *dsbA* from *Escherichia coli* O157:H7 results in decrease bacterial motility, inability to form biofilms, and attenuated virulence likely attributed to deficiencies in colonizing the host (Lee *et al.*, 2008). The absence of *dsbA* from *Pseudomonas aeruginosa* fails to form its Type III Secretion System (T3SS), has decreased bacterial motility, and is unable to replicate intracellularly (Ha *et al.*, 2003). Additionally, other prominent human pathogens like *Burkholderia cepacia*, *Burkholderia pseudomallei*, *Corynebacterium diphtheriae*, *Francisella tulerensis*, and *Mycobacterium tuberculosis*, to name only a few, have had their Dsb proteins directly linked to virulence (Hayashi *et al.*, 2000; Qin *et al.*, 2008; Premkumar *et al.*, 2013; Ireland *et al.*, 2014; Reardon-Robinson *et al.*, 2015). The highly conserved active sites of Dsb proteins and the critical role they have in ensuring virulence factors (including components of the T4BSS complex) are properly folded and biologically active make them an attractive target for further research into how they can be utilized as therapeutic alternatives to antibiotics. Targeting *C. burnetii* T4BSS directly or indirectly through proteins essential for T4SS formation would be especially beneficial for Q fever since the survival of the pathogen, intracellularly, is entirely reliant on a functional T4BSS (Beare *et al.*, 2011).

Bioinformatic analysis of the amino acid sequences of the *C. burnetii* protein components that comprise the T4BSS complex reveals that of all 24 T4BSS protein components, 13 are putative substrates of Dsb proteins since they contain 2 or more cysteine residues (Table 2-1). The main component of the T4BSS core complex that spans the periplasm is IcmE/dotG (CBU_1627), has 15 cysteine residues of a total 1039 amino acids residues. Of the 13 T4BSS components that are putative Dsb protein substrates, 7 are predicted to have transmembrane domains or be anchored in either the inner or outer membrane, making them likely substrates of a periplasmic Dsb protein.

An innovative approach to advance treatment options for Q fever is to identify and understand the role of Dsb proteins in Q fever pathogenesis. This strategy has the potential to provide novel therapeutic targets for other difficult to treat intracellular bacterial pathogens as well. One of the immunodominant proteins identified early in *C. burnetii* studies was named the *Coxiella* outer membrane protein 1 (Hendrix *et al.*, 1993). The presence of the hallmark CXXC motif in the Com1 amino acid sequence is indicative of the active site of Dsb proteins and suggested that Com1 may function as a Dsb protein, however, no additional work has been published to validate the biochemical function of Com1. Prior to the identification of *com1* through the initial cloning and sequencing study in 1993 by Hendrix *et al.*, several immunological studies of Green Monkey Kidney cells, guinea pigs, and rabbits infected with *C. burnetii* identified an unknown protein that migrated at 27 kDa when analyzed by Western blot (Müller *et al.*, 1987; Schmeer, 1988; Hendrix *et al.*, 1990). Subsequently, the 27 kDa immunodominant protein identified by Hendrix *et al.* (1990) was identified as Com1,

although, there is no certainty that the earlier 27 kDa proteins identified on the Western blots were Com1 (Hendrix *et al.*, 1990). Three decades after the 27 kDa immunodominant protein was first identified, the 27 kDa Com1 protein has been identified as an immunodominant protein for humans infected with *C. burnetii*, highlighting the importance of this protein for patients afflicted with Q fever (Sekeyová *et al.*, 2008). The small, but relevant literature surrounding Com1, spurred groups to explore if Com1, acting as an antigen, could be a basis for vaccine development. Early studies discovered that immunization of mice with Com1 generated only weak protection against a challenge with virulent *C. burnetii* strain Nine Mile phase I (NMI) (Zhang and Samuel, 2003). One of the safest and most efficacious vaccines against *C. burnetii*, Q-Vax (CSL, Australia) uses formalin-inactivation of the virulent NMI strain (Ackland *et al.*, 1994). Previous studies had determined that the incomplete O-LPS, found in the avirulent Nine Mile phase II (NMII) strain, did not induce an immune response or illicit protection against NMI that had the full LPS O-antigen (Zhang *et al.*, 2007). Additional attempts have been made to capitalize on *C. burnetii* LPS-targeted peptide mimics that may illicit stronger protection against subsequent challenge trials and these studies have shown promise through increased protection against challenges with NMI for immunized mice (Peng *et al.*, 2012).

Results

Identification and phylogenic analysis of genes encoding putative disulfide bond proteins

The first sequence analysis of *com1* from *C. burnetii* NMI, found a feature of Com1 amino acid sequence that aligned with the active sites of protein disulfide isomerase

(PDI) from rat and DsbA from *E. coli* (Hendrix *et al.*, 1993). The presence of this Dsb protein catalytic active site in Com1, which has the conserved Cys-X-X-Cys active site motif characteristic of Dsb proteins, where two cysteine residues are separated by two amino acids, strongly suggested that Com1 belongs to the Dsb protein family (Hendrix *et al.*, 1993). Annotations from the subsequent analysis of genomic sequencing of *C. burnetii* NMI identified other putative proteins of the Dsb family of proteins, including DsbA, DsbB, and DsbD (Seshadri *et al.*, 2003). Given the lack of genomic sequences available when *com1* was identified, there were numerous limitations to the insights that analysis of the DNA and protein sequences could provide about the structure and likely functional Dsb protein class of Com1.

Alignments of amino acid sequences of Com1 with *E. coli* DsbC and *Legionella pneumophila* DsbA2 (Com1-like) reveal the following results (34% identity, 49% positive, 10% gaps for DsbC and 49.34% identity, 71% positive, 3% gaps for DsbA2) (Figure 2-1). A key feature noted in the alignment with *E. coli* DsbC and *L. pneumophila* DsbA2 proteins is the presence of a dimerization domain, shared by Com1. Functional and structural studies of *E. coli* DsbC and *L. pneumophila* DsbA2 proteins have revealed these functional proteins exists as homodimers and function as protein disulfide bond isomerases, a proofreading class of Dsb proteins (Missiakas *et al.*, 1994; Shevchik *et al.*, 1994; Zapun *et al.*, 1995; Kpadeh *et al.*, 2013; Kpadeh *et al.*, 2015). To obtain a broader perspective of how Com1 compares to other similar proteins in bacterial pathogens, the Com1 amino acid sequence was compared with Clustal Omega analysis of DsbA oxidoreductase proteins, DsbC PDI proteins, Com1 and Com1-like proteins in bacteria

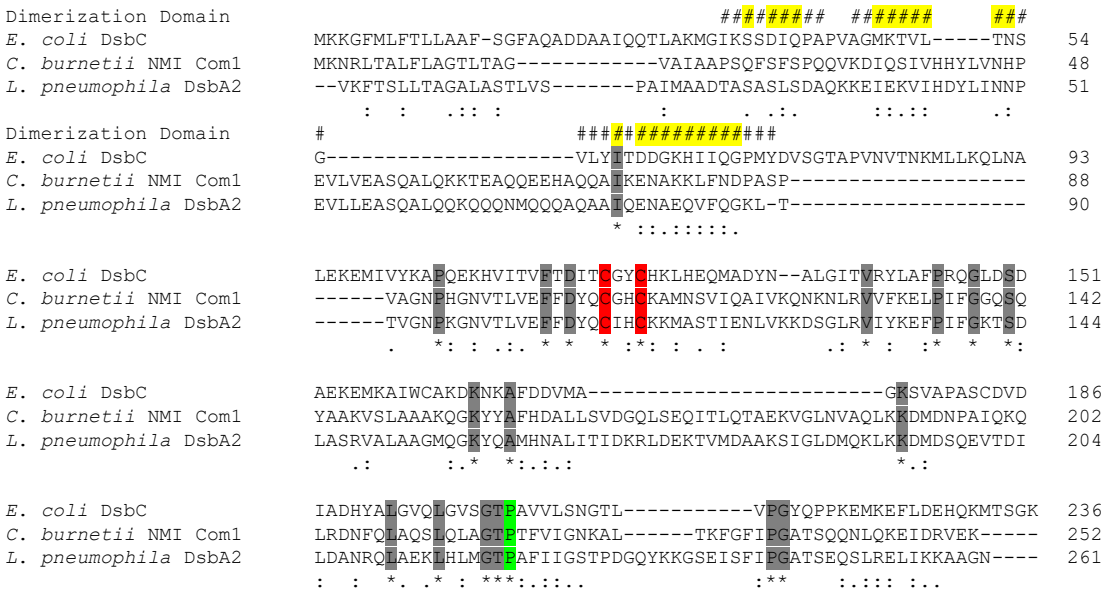


Figure 2-1. Protein sequence alignments of *C. burnetii* Dsb protein Com1. A. Alignments of amino acid sequences of *C. burnetii* Com1 with DsbC and Com1 proteins of *E. coli* and *L. pneumophila*, respectively, with identical residues highlighted in dark gray. The active site, CXXC, is highlighted red. The conserved dimerization domain of DsbC is indicated with # with highly and weakly similar residues of the dimerization domain are highlighted in yellow. Symbols below the alignments indicate identical residues (*), highly similar residues (:), and weakly similar residues (.). The proline residue conserved among these proteins that is situated opposite the CXXC active site in *E. coli* is highlighted in green.

with T4ASS and T4BSS complexes (encoded on the genome), with the exception of *E. coli* strain K-12: (*Agrobacterium fabrum s*train C58, *Bartonella henselae* strain Houston-1, *Brucella lelintensis* bovine 1 strain 16M, *C. burnetii* strain RSA 493 [NMI], *Escherichia coli* strain K-12 substrain MG1655, *Francisella tularensis* subsp. Tularensis SCHU S4, *L. pneumophila* subspecies pneumophila strain Philadelphia 1, *Rhodopseudomonas palustris* strain CGA009, *Rickettsia typhi* strain Wilmington). Phylogenic analysis of the different Dsb protein families from bacteria with T4BSS complexes reveals that the Com1 and Com1-like proteins across all bacteria cluster together from the same main branch, while DsbA and DsbC conservation is dependent upon the bacteria (Figure 2-2). It should be noted that *C. burnetii* strains lacks any ortholog of DsbC and does not encode redundant proteins that can serve or partially serve the functions of DsbA and DsbC. In the case with *E. coli*, which clusters separately from the other bacteria, it encodes multiple orthologs of DsbA and DsbC proteins that provide a degree of redundancy. This suggests that the bacteria that are reliant upon a T4BSS for replication have conserved differences in DsbA compared to *E. coli* that does not require a T4ASS for replication, but rather can make use of the plasmid-encoded T4ASS for bacterial conjugation (Yeo *et al.*, 2003, Durand *et al.*, 2010).

The trove of genomic and proteomic data currently available has provided new insights into structural and phylogenic properties of Com1 that require functional confirmation. Using published X-ray crystal structures of *E. coli* DsbC (PDB accesssion number: 1EEJ), predicted structures of Com1 have been generated with PredictProtein.org (Figure 2-3) (McCarthy *et al.*, 2000; Burnhofer *et al.*, 2021). Key elements of this predicted structure

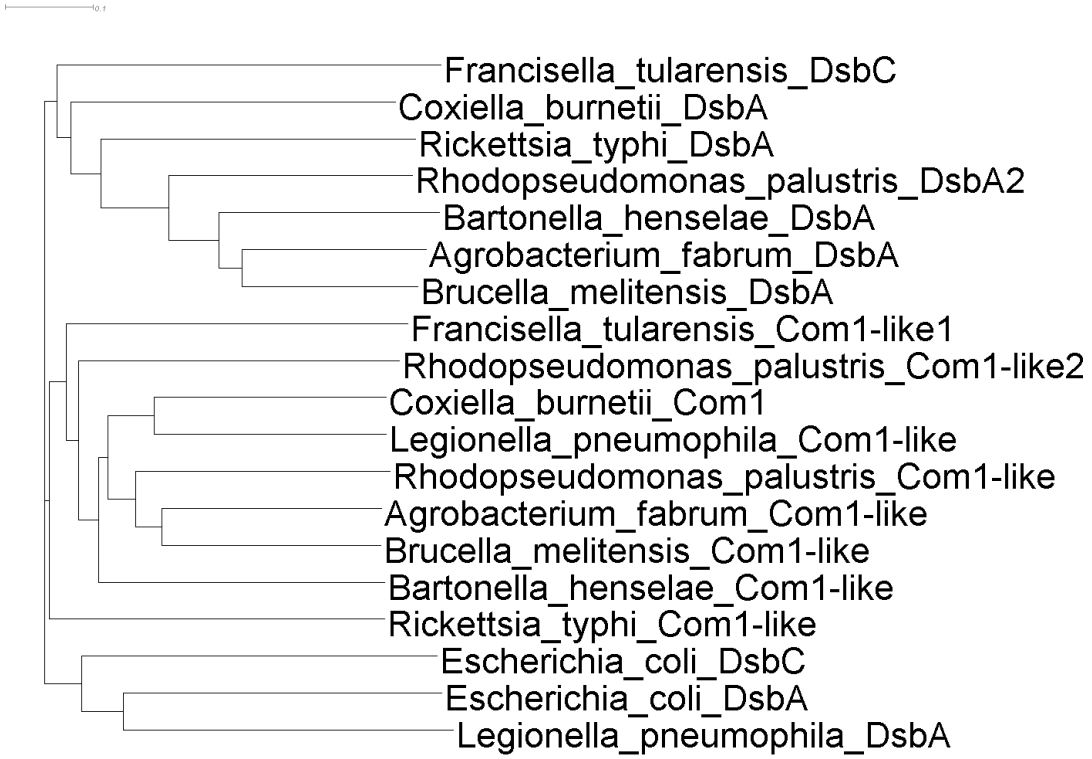


Figure 2-2. Phylogenetic analysis from Clustal Omega analysis of DsbA, DsbC, and Com1-like proteins. Proteins from bacteria encoding a T4ASS or T4BSS compared to *E. coli* strain K-12 substrain MG1655 (*Agrobacterium fabrum* AAK86609.1 and AAK87124.2, *Bartonella henselae* CAF27265.1 and CAF27606.1, *Brucella melitensis* AAL52621.1 and AAL52241.1, *Coxiella burnetii* AAO90419.2 and AAO91401.1, *Francisella tularensis* YP_169698.1 and YP_170079.1, *Legionella pneumophila* YP_094177.1 and YP_095867.1, *Rhodopseudomonas palustris* CAE29928.1 and CAE27883.1, *Rickettsia typhi* AAU03589.1 and AAU03894.1).

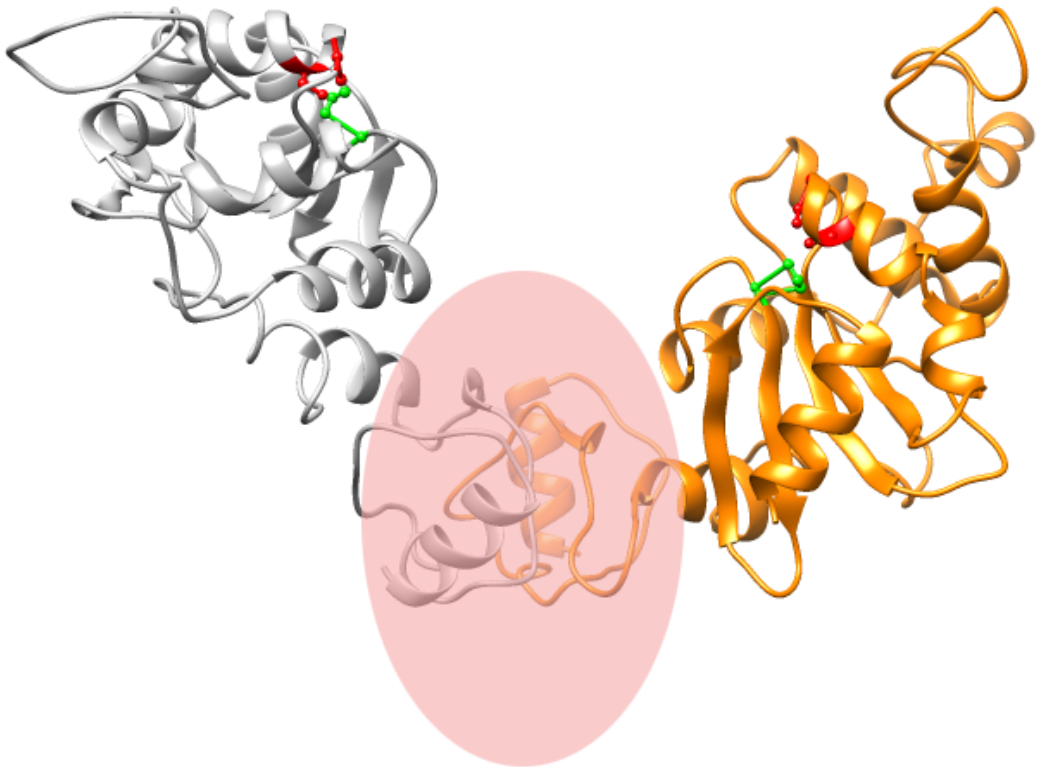


Figure 2-3. Predicted structure of Com1. Predictive structure was modeled on the crystal structure of *E. coli* DsbC (McCarthy *et al.*, 2000) 1EEJ that aligns with positions 38-251 of *C. burnetii* NMI Com1. The two monomers of the homodimeric Com1 are colored silver and orange. The two cysteine residues in the active site are red, and the green residues represent the conserved proline residue in Com1 proteins, dimerization domain is highlighted by the pink oval. Predicted structure generated with PredictProtein (Bernhoffer *et al.*, 2021) and modified with colors with UCSF Chimera version 1.11 (Pettersen *et al.*, 2004).

of a Com1 homodimer have the CXXC active site opposite a conserved proline residue (highlighted in green) that is conserved across DsbA and DsbC proteins (Kadokura *et al.*, 2004; Ren and Bardwell, 2011; Kpadeh *et al.*, 2013). The region at the center of the two monomers highlighted in pink is the predicted dimerization domain that allows for two monomers to interact and form a stable protein complex. The two red residues on each monomer represent the two cysteine residues of the active site (Zapun *et al.*, 1995).

The importance of the conserved proline residue opposite the active site was discovered when a million mutants were generated by UV-light exposure and screened for defects in Dsb activity using blue/white β-galactosidase activity that results in blue or white colonies (Kadokura *et al.*, 2004). This same study identified one of their mutants had an unexpected phenotype. In this mutant, when the proline in *E. coli* DsbA at position 151 was substituted with a threonine, Western blot migration with DsbA-specific primary antibodies detected DsbA bands migrating at numerous sizes when the protein samples were run under non-reducing conditions. If the protein samples were exposed to reducing agents, only one band would be visible on the Western blots. By sequencing the protein bands under non-reducing conditions, they discovered that this amino acid substitution at this conserved proline residue resulted in DsbA being covalently bound to the protein substrates. The amino acid sequence alignments of Com1 in Figure 2-1 reveal that proline is also conserved and is at position 219 of Com1.

Determining enzymatic properties of Com1

Based on sequence alignments and the presence of the CXXC active site motif

characteristic of Dsb proteins, Com1 was thought to be a putative oxidoreductase Dsb protein, however, Com1 was not assayed for functional oxidoreductase activity (Hendrix *et al.*, 1993). Unpublished efforts by Dr. Min Pannella in Dr. Guoquan Zhang's *Coxiella* laboratory, then at the University of Missouri – Columbia, successfully cloned *com1* without the predicted signal sequence from NMI into the pET28B expression vector and used an insulin reduction assay to produce preliminary results indicating that Com1 has functional oxidoreductase activity of a Dsb protein (Pannella and Zhang, unpublished data, Holmgren, 1979). These unpublished, preliminary results were expanded upon in this current study utilizing the same recombinant Com1 expression vector made by Dr. Pannella, along with the recombinant *E. coli* DsbA expression vector made by Dr. Pannella. An additional recombinant Com1 expression vector which included the signal sequence upstream of the 6x-histidine tag, was constructed introducing the P219T point mutation that changes the conserved *cis*-proline residue opposite the active site at position 219 to a threonine (Table 2-1). Experimental studies in *E. coli* and *L. pneumophila* DsbA and DsbA2, respectively, have determined the essential role of that *cis*-proline in resolving the disulfide bond catalyzed by Dsb proteins (Kadokura *et al.*, 2004, Ren and Bardwell, 2011; Kpadeh *et al.*, 2013). One key observation after the discovery of the DsbC protein of *E.coli* in 1994 was the difference in enzyme kinetics during insulin reduction (Missiakas *et al.*, 1994; Zapun *et al.*, 1995). Zapun *et al.*, discovered that, unlike DsbA, DsbC functions as a homodimer, bound together by a dimerization domain and that catalytic activity produced higher rates of insulin reduction. When comparing enzymatic profiles of purified *C. burnetii* Com1 and Com1 P219T with purified *E. coli* DsbA, the same higher rates of insulin reduction

Table 2-1. List of strains and plasmids

Strain or plasmid	Description	Reference/Source
C. burnetii strains		
RSA 493 Nine Mile I		
RSA 439 Nine Mile II		
RSA 439 NMII Empty	pJBProBA-Kan-Empty	This study
RSA 439 NMII 6xH WT	pJBProBA-Kan-6xHWTCom1	This study
RSA 439 NMII 6xH P219T Com1	pJBProBA-Kan-6xHP219TCom1	This study
E. coli strains		
BL21-AI™	F- *ompT hsdS*$_B$ (r_B-m_B-) *gal dcm araB*::*T7RNAP-tetA*	Intact Genomics, Inc.
ig™ 5a	Φ80 Δ(*lacZ*)M15 *fhuA2* Δ(*argF-lacZ*)U169 *phoA glnV44 gyrA96 recA1 relA1 endA1 thi-1hsdR17*	Invitrogen
TOP10™	F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
Stellar™	F-, *endA1*, *supE44*, *thi-1*, *recA1*, *relA1*, *gyrA96*, *phoA*, Φ80d *lacZ*Δ M15, Δ(*lacZYA-argF*) U169, Δ(*mrr-hsdRMS-mcr*BC), Δ*mcrA*, λ-	Takara Bio USA, Inc.
NPO0145	BL21-AI™ + pENTR-FLNMI*com1*	This study
NPO0146	BL21-AI™ + pENTR-FLNMIP219T*com1*	This study
NPO0168	BL21-AI™ + pDEST17-FLNMI*com1*	This study
NPO0169	BL21-AI™ + pDEST17-FLNMIP219T*com1*	This study
NPO0206	Stellar™ + pDEST17-ss-6xH*com1*	This study
NPO0207	Stellar™ + pDEST17-ss-6xH*com1* P2197	This study
RGP665	ER1821 Δ*dsbC* + pPDI	Ren and Bardwell, 2011
NPO0221	RGP665 + pJBProBA-Kan-Empty	This study
NPO0222	RGP665 + pJBProBA-Kan-6xH-WTCom1	This study
NPO0223	RGP665 + pPDI + pJBProBA-Kan-6xH-P219TCom1	This study
Plasmids		
pENTR™/SD/D		Invitrogen
pENTR-FLNMI*com1*	Full length NMI *com1*	
pENTR-FLNMIP219T*com1*	Full lengths NMI *com1* P219T	
pET28-Δss-Com1	NMI c*om1* minus signal sequence	Pannella M,
pET28-Δss-DsbA	*E. coli dsbA* minus signal sequence	Pannella M,
pDEST17		Invitrogen
pDEST17-ss-6xH*com1*	Signal sequence-6xHistidine-WT NMI *com1*	
pDEST17-ss-6xH*com1* P219T	Signal sequence-6xHistidine-WT NMI *com1* P219T	
pJBProBA-2xHA-Amp		Gifted by Dr.Paul Beare
pJBProBA-Kan-Empty		This study
pJBProBA-Kan-6xH-WTCom1	Signal sequence-6xHistidine-NMI WT *com1*	This study
pJBProBA-Kan-6xH-P219TCom1	Signal sequence-6xHistidine-NMI *com1* P219T	This study
pPDI detector		Ren and Bardwell, 2011

with Com1 compared to DsbA are observed (Figure 2-4). This is further biochemical evidence suggesting that Com1 is a functional protein disulfide isomerase.

Functional assessment of Com1 in E. coli dsbC mutant

In order to verify that Com1 functions as a protein disulfide isomerase, additional assays are required. Since stable genetic manipulation of *C. burnetii* is a relatively recent development, there are molecular tools that have not yet been adapted to function for studies in *C. burnetii*. One of these molecular tools is used to determine whether a Dsb protein is a functional protein disulfide isomerase. To answer if Com1 is a protein disulfide isomerase, a full-length recombinant protein was engineered, with signal sequence intact and cloned it into the pJBProBA-Kan expression vector. To test this expression plasmid, the *E. coli dsb* mutant strain library generously provided by Dr. Bardwell that contains a protein disulfide isomerase detection plasmid, pPDI was used (Ren and Bardwell, 2011). The pPDI vector contains a mutated β-lactamase that has two additional cysteine residues that do not affect enzyme function if the correct disulfide bond is formed between two native cysteine residues. Therefore, if a protein disulfide isomerase is present, the correct disulfide bonds of β-lactamase will be formed allowing for bacterial colonies to grow in the presence of inhibitory concentrations of ampicillin. When recombinant Com1 and Com1 P219T proteins are over-expressed in the *E. coli dsbC* mutant strain, only induced expression of *com1* is able to a show bacterial growth in the presence of inhibitory ampicillin (Figure 2-5). This complementation study also reveals that rescue with Com1 P219T displays an impaired rescue phenotype, which is

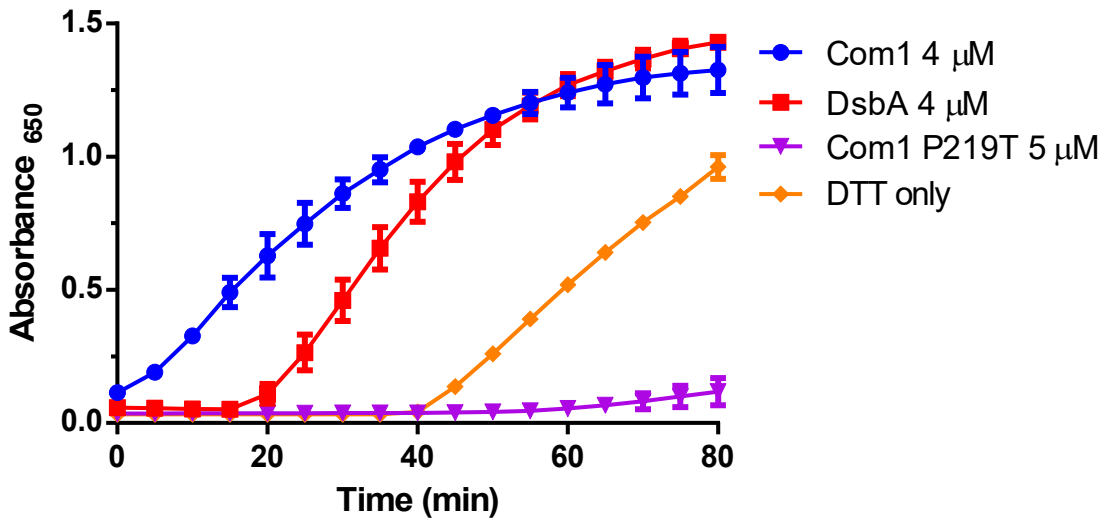


Figure 2-4. Insulin disulfide reduction assay of *C. burnetii* Com1, Com1 P219T, and *E. coli* DsbA. Demonstrates oxidoreductase activity of the proteins. Note that DTT is present in all reaction mixtures, but DTT alone is able to reduce of the disulfide bonds between insulin chains with after a 40 minute delay as observed in studies on thioredoxin and *E. coli* DsbA (Holmgren, 1979 and Bardwell *et al.*, 1991). Also note that DTT treatment in the presence Com1 P219T is unable to reduce insulin. Error bars represent standard deviation of three replicates.

expected if β-lactamase is irreversibly binding to Com1 P219T. The uninduced controls in the top panel of Figure 2.5, only display growth when cultures are spotted on the plate undiluted for the empty vector and diluted 10^{-3} for the WT *com1* vector on plates with 1 g/L ampicillin. Comparatively, under IPTG induction, bacteria containing the WT *com1* display growth when diluted as far as 10^{-3} on plates with up to 3 g/L ampicillin. This indicates that under IPTG induction, Com1 displays sufficient protein disulfide isomerase activity to correctly fold the β-lactamase expressed from the pPDI vector. Collectively, these data definitively show that Com1 has the enzymatic properties of a protein disulfide isomerase, which is clearly demonstrated in the ability of Com1 to rescue the *E. coli* Δ*dsbC* protein disulfide isomerase mutant strain as is evidenced by bacterial growth in the presence of inhibitory concentrations of ampicillin.

Determination of in vivo redox state of Com1 in C. burnetii NMII

Dsb proteins provide essential functions for bacterial cells by catalyzing the creation of disulfide bonds of periplasmic proteins with two or more cysteine residues (DsbA) and rearranging those disulfide bonds of proteins with more than two cysteine residues (DsbC). Collectively, Dsb proteins allow for proper structural folding of periplasmic proteins to carry out their functional roles (Raina and Missiakas, 1997). Since DsbA and DsbC proteins have different functions, the cysteine residues of the active sites for each protein exists in different states of reduction and oxidation (redox); cysteine residues of DsbA proteins are oxidized, while cysteine residues of protein disulfide isomerase

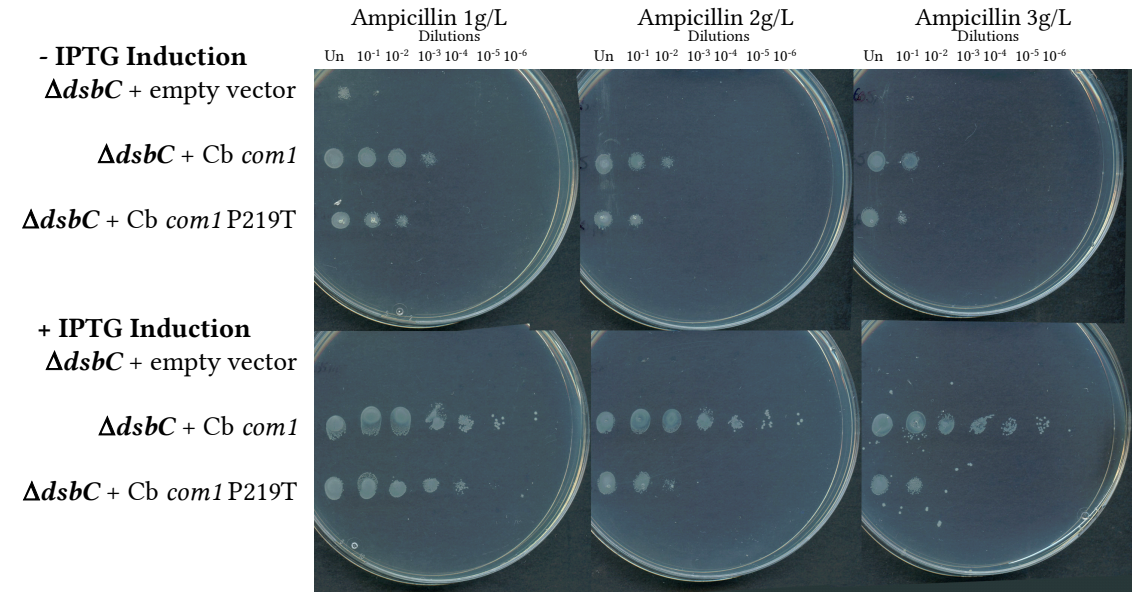


Figure 2-5. Complementation of *E. coli dsbC* mutant strain with recombinant *C. burnetii* Com1 and Com1 P219T. Serial 10-fold dilutions of uninduced (top panel) and 0.5 mM IPTG induced (bottom panel) cultures of *E. coli* Δ*dsbC* strain RGP665 (bold gene designation refers to *E. coli* strain genotype) with pPDI detection plasmid transformed with pJBProBA-Kan-Empty (empty vector), pJBProBA-Kan-6xH-WTCom1 (Cb Com1), and pJBProBA-Kan-6xH-P219TCom1 (Cb Com1 P219T), plated on increasingly prohibitive concentrations of ampicillin (1 g/L, 2 g/L, and 3 g/L).

DsbC proteins are reduced (Joly and Swartz, 1997; Denocin *et al.*, 2013). To verify Com1 functions in *C. burnetii* NMII is a protein disulfide isomerase, the *in vivo* redox state of the protein has been determined by use of the thiol agent, 4'-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) that increases the molecular mass of Dsb proteins by 490 Da *in vivo* by binding to reduced thiol groups (Hansen and Winther, 2009). Cultures of transformed NMII with pJBProBA-Kan-6xH-WTCom1 and pJBProBA-Kan-6xH-P219TCom1 were grown for 6 days, then induced with 0.5 mM IPTG for 16 hours and cultures were processed as described in the methods (Ren and Bardwell, 2011; Denoncin *et al.*, 2013). Samples were visualized by Western blot with anti-6x histidine primary murine-derived IgG2A antibodies (1:3000) (Invitrogen, USA) and anti-mouse IgG2A goat-derived HRP conjugated secondary antibodies (1:2000) (Invitrogen, USA). Analysis of the blot reveals that Com1 WT (lanes 1-3) under reducing conditions of 1 mM DTT, is approximately midway between the fully reduced and fully oxidized purified Com1 protein (Figure 2-6). This is indicative of two of the four cysteine residues of the homodimeric protein being reduced and two cysteine residues being oxidized (Joly and Swartz, 1997). This is further evidence that supports Com1 functions in the periplasmic space of *C. burnetii* NMII as a protein disulfide isomerase.

Identification of Com1 substrates by mass spectrometry

Having determined the enzymatic properties and function of Com1 as the only identified protein disulfide isomerase in the *C. burnetii* genome, it was necessary to

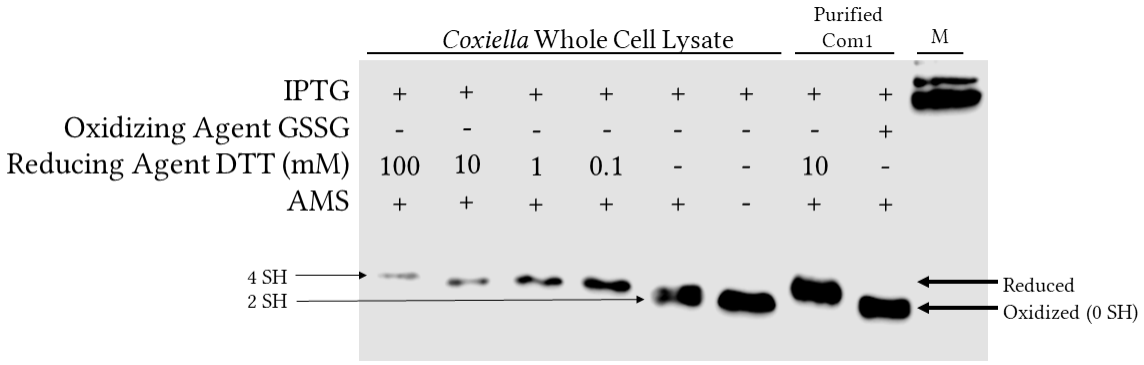


Figure 2-6. *In vivo* redox state of recombinant 6x Histidine tagged Com1. Cultures of *C. burnetii* treated with 100, 10, 1, and 0.1 mM DTT to reduce proteins *in vivo* prior to extracting proteins from whole cell lysate and visualizing by Western blot.

identify what proteins are the substrates of the Com1 enzyme. Using the recombinant Com1 P219T mutant cloned in the pJBProBA-Kan vector, *C. burnetii* NMII was transformed and the bacteria cultured in 1X ACCM-D without proline to select for the transformants containing the plasmid. Cultures were expanded to 4 L and grown for 6 days and induced with 0.5 mM IPTG for 16 hours and harvested. Care was taken to extract proteins from the outer membrane and periplasmic space by well established methods (Quan *et al.*, 2013). Protein extracts were purified with Ni-NTA metal chromatography and fractions containing the Com1 protein were further processed, cleaned, and trypsin digested at the MU Gehrke Proteomics Center and analyzed by trapped ion mobility (tims) time of flight (TOF) liquid chromatography (LC) dual mass spectrometry (timsTOF LC-MS/MS) in the timsTOF-Pro (Bruker, USA). Protein hits were screened against the UniProt *C. burnetii* database by PEAKS Studio (Bioinformatic Solutions, Inc, Canada). Analysis of the data revealed 64 *C. burnetii* proteins, excluding Com1 (Table 2-2). Half of the replicates of the P219T substrate capture and protein extracts from the recombinant Com1 WT cultures to use as negative controls to screen out non-specific proteins hits have been processed and half are currently being processed for submission to the MU Gehrke Proteomics Center in order to statistically analyze the data and provide reliable results of verified hits. The results of proteins identified in both replicates of the Com1 WT cultures that have been processed and analyzed are presented in the Appendix (Table A-1). The results of the Com1 WT controls indicate which proteins have also been identified in the substrate capture samples, but the full analysis of these controls in relation to the hit frequencies obtained for each protein hit cannot be completed until all replicates are processed.

Table 2-2. List of proteins identified from Com1 P219T substrate capture.

Protein Description	Locus Tag	Accession	Predicted Localization	Cysteines	Hit Frequency
Uncharacterized protein	CBU_0089a	B5QS73	Unknown	2	9
Tol-Pal system protein TolB	CBU_0090	Q83F59	Periplasm **(P)**	0	7
Uncharacterized exported protein	CBU_0110	Q83F41	**Inner membrane (IM)/P** ǂ	7	8
C40 family peptidase	CBU_0215	Q83EU4	P	2	2
50S ribosomal protein L7/L12	CBU_0229	P0C8S3	**Effector (E)** ǂ	0	2
Tuf-2 elongation factor Tu	CBU_0236	Q83ES6	**Cytoplasm (C)** ǂ	2	5
30S ribosomal protein S17	CBU_0247	Q83ER7	C	1	2
30S ribosomal protein S13	CBU_0260	P59753	C	1	6
Single-stranded DNA-binding protein	CBU_0271	Q83EP4	**Outer membrane (OM)** ǂ	0	3
50S ribosomal protein L33	CBU_0290	Q83EM5	C	0	2
OmpA-like outer membrane protein	CBU_0307	Q83EL2	**OM** ǂ	0	17
30S ribosomal protein S16	CBU_0445	Q83E83	C	1	4
Histone-like protein Hq1	CBU_0456	Q45881	Unknown	0	3
50S ribosomal protein L32	CBU_0491	Q83E41	C	0	12
Uncharacterized protein	CBU_0516a	B5QS96	P	7	10
ComE competence operon protein 1	CBU_0532	Q83E05	**P** ǂ	0	8
Uncharacterized protein	CBU_0562a	B5QS99	P	7	15
OmpH outer membrane protein	CBU_0612	Q83DT1	**P** ǂ	1	32
FKBP-type peptidyl-prolyl cis-trans isomerase*	CBU_0630	P51752	OM	0	64
Uncharacterized protein	CBU_0632	Q83DR4	**C** ǂ	1	3
Uncharacterized exported protein	CBU_0731	Q83DJ9	P	6	7
Acriflavin resistance periplasmic protein	CBU_0754	Q83DH7	IM	1	4
Periplasmic serine endoprotease DegP-like	CBU_0755	Q83DH6	P	0	34
Uncharacterized protein	CBU_0802	Q83DD6	P	0	2
Enhanced entry protein	CBU_0915	Q83D29	**P** ǂ	5	7
UPF0422 protein	CBU_0937	Q83D09	**OM** ǂ	5	7
Uncharacterized exported protein	CBU_1095	Q83CL9	**P** ǂ	5	2
Alpha-acetolactate decarboxylase	CBU_1097	Q83CL7	Unknown	0	4
Uncharacterized membrane associated protein	CBU_1134	Q83CI1	IM/P	3	2
Uncharacterized exported protein	CBU_1135	Q83CI0	P	4	2
Uncharacterized protein	CBU_1173	Q83CE6	P	4	7
Glycine-rich RNA-binding protein	CBU_1183	Q83CD7	**Unknown** ǂ	0	22
Outer-membrane lipoprotein carrier protein	CBU_1190	P39917	**OM** ǂ	0	4
Serine-type D-Ala-D-Ala carboxypeptidase	CBU_1261	Q83C68	P	2	3
Chaperone protein DnaK	CBU_1290	O87712	**C** ǂ	2	11
50S ribosomal protein L35	CBU_1324	Q83C12	C	0	6
Translation initiation factor IF-3	CBU_1325	Q83C11	**C** ǂ	1	5
Uncharacterized exported protein	CBU_1366	Q83BX1	P	1	3
Uncharacterized exported protein	CBU_1404	Q83BU6	P	7	14
17 kDa common-antigen	CBU_1425	Q83BS7	OM/P	3	5

Protein Description	Locus Tag	Accession	Predicted Localization	Cysteines	Hit Frequency
Uncharacterized protein	CBU_1429a	B5QSD3	P	8	7
HupB DNA-binding protein HU	CBU_1464	Q83BN9	**OM** ǂ	0	3
Carboxy-terminal processing protease	CBU_1538	Q83BH0	IM/P	0	17
30S ribosomal protein S21	CBU_1593	Q83BB9	C	1	3
DotH (IcmK) T4BSS component	CBU_1628	Q83B85	OM	1	2
DotA, T4BSS component	CBU_1648	Q83B67	IM/P	7	22
IcmX, T4BSS component	CBU_1652	Q83B63	**P** ǂ	0	13
CBS domain containing protein	CBU_1664	Q83B51	C	1	2
Uncharacterized protein	CBU_1705	Q83B15	**Unknown** ǂ	0	20
Alkyl hydroperoxide reductase	CBU_1706	Q83B14	**C** ǂ	3	6
Chaperonin GroEL*	CBU_1718	P19421	**E** ǂ	2	14
Co-chaperonin GroES*	CBU_1719	P19422	**C** ǂ	0	41
Aconitate hydratase	CBU_1720	Q83B05	C	12	2
Uncharacterized exported protein	CBU_1735	Q83AZ1	P	1	3
Glyceraldehyde-3-phosphate dehydrogenase*	CBU_1783	Q83AU5	**C** ǂ	4	7
Superoxide dismutase [Cu-Zn] SodC*	CBU_1822	Q83AQ8	IM/P	2	6
Uncharacterized exported protein	CBU_1847	Q83AN5	P	0	8
Uncharacterized protein	CBU_1847b	B5QSG2	P	6	8
Uncharacterized exported protein	CBU_1869	Q83AL4	P	7	5
Non-proteolytic protein, peptidase family M16	CBU_1901	Q83AI5	P	0	4
Com1 *Coxiella* outer membrane protein 1	CBU_1910	H7C7D7	P	2	78
Uncharacterized exported protein	CBU_1984	Q83AC0	P	8	19
ATP-dependent protease ATPase subunit HslU	CBU_2012	Q83A94	**C** ǂ	0	4
Uncharacterized exported protein	CBU_2072	Q83A39	P	4	6
Thioredoxin	CBU_2087	Q83A24	C	2	3

Table 2-2. List of Proteins Identified from Com1 P219T Substrate Capture. List comprises 65 proteins without exclusion of proteins from the negative control. Predicted localization in bold and marked with, ǂ , indicates localization has been previously determined by Flores-Ramirez et al., 2014. All other predicted localization was made using PSORTb and PROTTER. Proteins hits likely identified due to natural immobilization on Ni-NTA resin are indicated by, *, based on similarity with known *E. coli* proteins (Bolanos-Garcia and Davies, 2006). Periplasm (P), effector (E), inner membrane (IM), outer membrane (OM), cytoplasm (C).

Of the 64 proteins hits, 41 hits have one or more cysteine residues, including the T4BSS proteins, DotA and DotH (IcmK), essential components for the formation of the T4BSS of *L. pneumophila* (Roy *et al.*, 1998). As found in a similar analysis of *L. pneumophila* with a substrate-capturing mutant of the *L. pneumophila* Com1-like protein, DsbA2, the initial analysis has also returned a mix of protein hits from the cytoplasm, inner membrane, periplasm, and outer membrane (Jameson-Lee *et al.*, 2011). Protein localization was determined by PSORTb v. 3.0.3, PROTTER, or based on experimental data by previous studies (Yu *et al.*, 2010; Flores-Ramirez *et al.*, 2014; Omasits *et al.*, 2014). Of those 41 interacting proteins identified in this study, 20 are predicted to be localized in the inner membrane, periplasm, or outer membrane by the presence of signal sequences. Seventeen of these proteins are unique to *C. burnetii* and have no currently known function. Of interest are the protein hits for CBU_0215, CBU_0915, CBU_1261, CBU_1822, which are predicted as a C40 family peptidase, enhanced entry protein, serine-type D-alanyl-D-alanine carboxypeptidase (DAP) virulence factor, and superoxide dismutase, respectively. C40 family peptidases like Spr (PA1198) and YdhO (PA1199) are thought to be involved in cell wall remodeling of *P. aeruginosa*, which also has a T4SS, however this peptidase is not part of the T4SS (Heywood and Lamont, 2020).

There is no currently published information on the *C. burnetii* putative C40 family peptidase, CBU_0215 to provide insight into the function it may serve apart from cell wall maintenance (Heywood *et al.*, 2020). The protein hits, CBU_0915 is annotated as having enhanced entry functions and has been identified in previous proteomic studies,

yet no data currently exists detailing its function (Skultety *et al.*, 2011; Flores-Ramirez *et al.*, 2014). The putative DAP protein encoded by CBU_1261 is of particular interest as studies in both *Brucella abortus* and *Francisella tularensis* show decreases in virulence when the genes encoding DAP are mutated (Kikuchi *et al.* 2006; Kijek *et al.*, 2019). The CU/Zn superoxide dismutase encoded by CBU_1822 has been well characterized in *E. coli* complementation studies and biochemical assays, though it has yet to be assayed in *C. burnetii* (Brennan *et al.*, 2015).

Although the current data set has yet to be completed, a deeper look at OmpH (CBU_0612) is required as it has, apart from Com1, one of the higher hit frequencies in two replicates of the P219T Com1. OmpH contains only one cysteine residue, located near the N-terminus of the protein in the predicted signal sequence, making it unlikely to be a substrate of Com1. Little structural homology exists for OmpH in current structural databases. The closest solved protein structure with an average model confidence score of 0.65 (16.3% amino acid sequence identity) is the 140 amino acid ATP synthase subunit B (6n2y.1.K) of *Bacillus* PS3, which aligns with amino acids 34-133 of OmpH (Figure 2-7). This structural alignment comprises a long helix structure (Figure 2-8). It should be noted that hit frequencies for OmpH are also high for both WT Com1 controls, suggesting this protein may non-specifically bind to Ni-NTA resin.

DotA (CBU_1648), in contrast to OmpH, only has high hit frequencies in the P219T

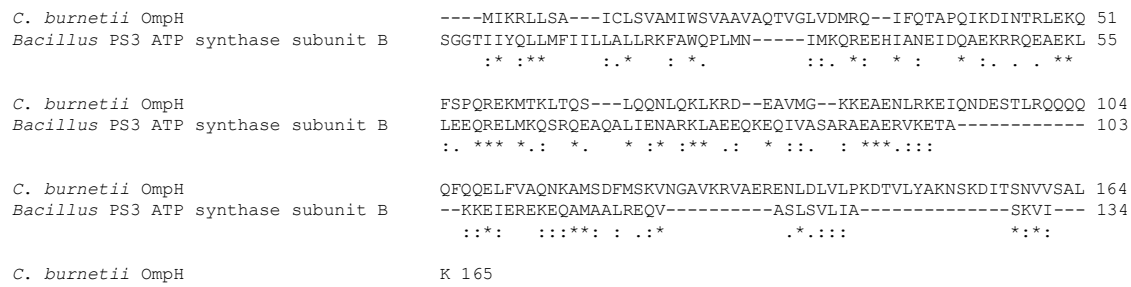


Figure 2-7. Protein sequence alignment of *C. burnetii* OmpH. Alignments of amino acid sequences of *C. burnetii* OmpH with ATP synthase subunit B (6n2y.1.k) protein of *Bacillus* PS3. Symbols below the alignments indicate identical residues (*), highly similar residues (:), and weakly similar residues (.).

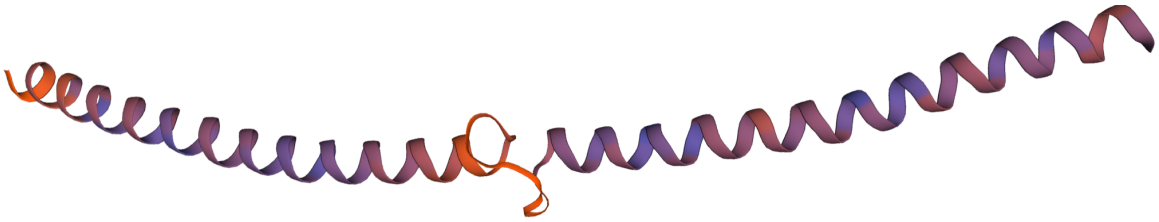


Figure 2-8. Predicted structure of OmpH. Predictive structure was modeled on the crystal structure of *Bacillus* PS3 ATP synthase subunit B (Guo *et al.*, 2019) 6n2y.1.K that loosely aligns with positions 1-165 of *C. burnetii* NMI OmpH. Predicted structure generated with SWISS-MODEL (Waterhouse *et al.*, 2018).

Com1 substrate capture results and low hit frequencies in both WT Com1 controls, suggesting that DotA is enriched by capture to P219T Com1. DotA shares 31% amino acid identity and 47% positive amino acids with DotA of *L. pneumophila*. Unfortunately, no solved structures exist for any predicated transmembrane DotA protein (Figure 2-9). Analysis of the DotA amino acid sequence for putative disulfide bonds reveals two possible bonds between C209 and C323 and C281 and C314 (Ferrè and Clote, 2005a; Ferrè and Clote, 2005b; Ferrè and Clote, 2006;). Structure prediction models only align with three transmembrane segments with no predicted involvement in disulfide bond formation, sharing 11% amino acid identity with the protein dispatched homolog 1 (7e2i.1.B) of humans with an average model confidence score of 0.28 (Figure 2-10).

Discussion

When bacterial pathogens have growth requirements beyond the traditional, well characterized aerobic and facultative anaerobes, or have additional levels of safety restrictions based upon ease of aerosolization and regulatory requirements, understanding the molecular mechanisms underlying basic biology and pathogenesis requires more time and effort. Such is the case with *C. burnetii*, the obligate intracellular bacterial cause of Q fever. Without a host-free culture method, progress in the Q fever field has been slow when compared to other bacterial pathogens. The advent of and continual improvements in axenic media for *C. burnetii* from the Q fever group at Rocky Mountain Laboratories have provided researchers with the tools to begin asking foundational questions about what bacterial genetic factors are required

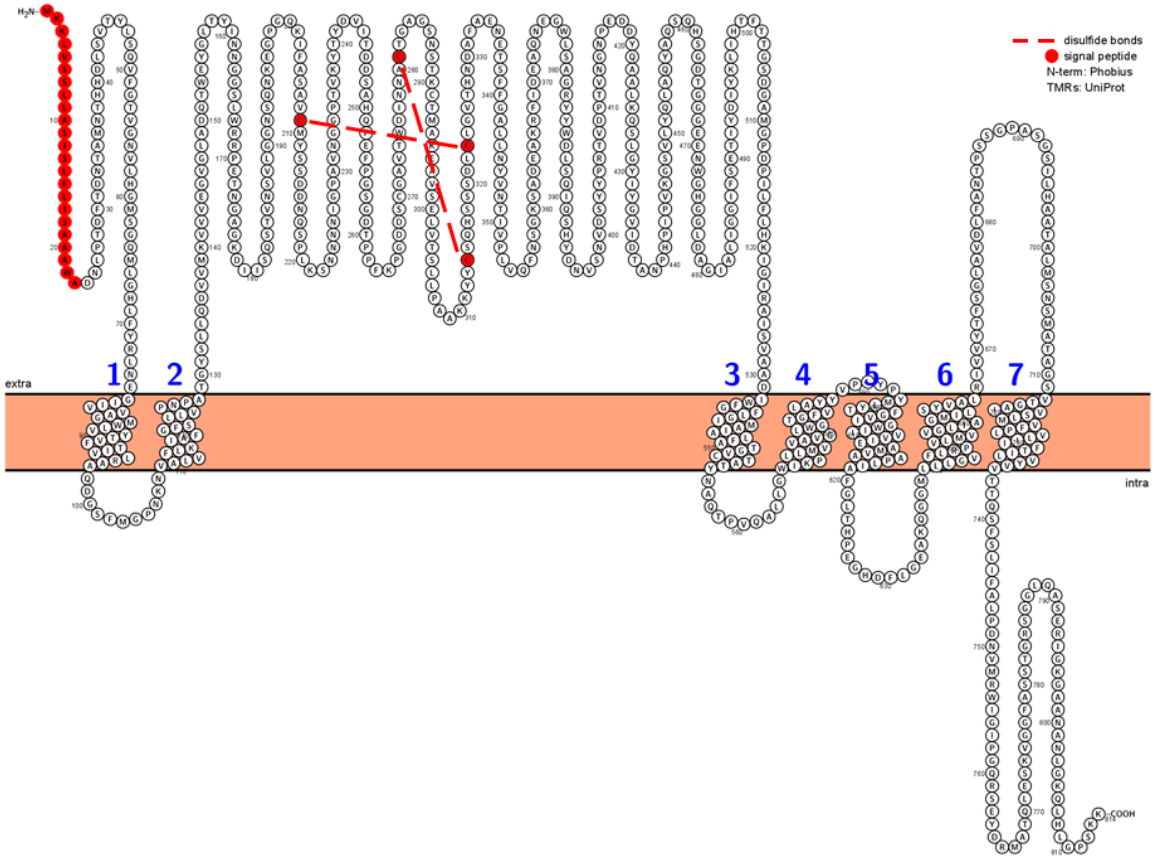
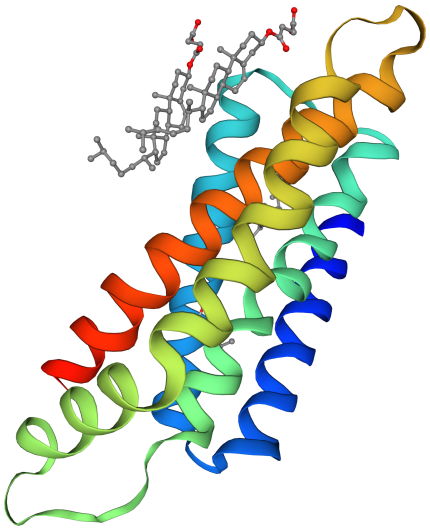


Figure 2-9. Predicted protein membrane orientation of *C. burnetii* DotA. Predicted disulfide bond generated with DiANNA (Ferrè and Clote, 2005a; Ferrè and Clote, 2005b; Ferrè and Clote, 2006;). Predicted transmembrane domains of DotA generated with PROTTER (Omasits *et al.*, 2014).

A.



B.

```
C. burnetii DotA                          AIALFATGVCTATYNAQTPVQALLGWIKPLLMVVAVGLWGTGFVLAYYVPLYPY------ 594
H. sapien Protein Dispatched Homolog 1    --------------------GTLIAMGLSVAVAFSVMLLTTWNIIISLYAIISIAGTIFV 40
                                                             :*:.    : :..:* *  *  ::     :

C. burnetii DotA                          MLYTFGVIGWIIVVIEAMVAAPLIAFGLTHPEG------HDFLGEAKQGGMLLLGVFLRP 648
H. sapien Protein Dispatched Homolog 1    TVGSLVLLGWELNVLESVTISVAVGLSVNFAVHYGVAYRLAPDPDREGKVIFSLSRVGSA 100
                                           : :: ::** : *:*::. :  :.:.:..             : :   :: *. .

C. burnetii DotA                          VLMVVGLIAGMILSYVALRIVVYTFSGLAVDLFANTPSSGPASGSILHAATALMSNSMAT 708
H. sapien Protein Dispatched Homolog 1    MAMAALTTFVAGAMMMPSTVLAYTQLGT-------------------------------- 128
                                          : *..          :   ::.**  *

C. burnetii DotA                          AGSVTGAIVSLMVFPLVLIIFTILVYVVTTQSFSLIFALPDNVMRWIGIPGQRSEYDRMA768
H. sapien Protein Dispatched Homolog 1    ----FMMLIMCISWAFATFFFQCMCRCL-------------------------------- 152
                                                 ::  : : :. ::*  :   :

C. burnetii DotA                          TQLESKVGGFASSTGRSGGLQASERIGKGAANANLGKQLHLGPSKK 814
H. sapien Protein Dispatched Homolog 1    ---------------------------------------------- 152
```

Figure 2-10. Protein sequence alignment of *C. burnetii* DotA and predicted structure. Alignments of amino acid sequences of *C. burnetii* Dot with Protein Dispatched Homolog 1 (7e2i.1.B) protein of *Homo sapiens* (Li *et al.*, 2021). Predicted structure generated with SWISS-MODEL (Waterhouse *et al.*, 2018). Symbols below the alignments indicate identical residues (*), highly similar residues (:), and weakly similar residues (.).

and how those factors interact with one another to influence bacterial growth and facilitate infection of host cells (Omsland *et al.*, 2011; Beare and Heinzen, 2014; Sandoz *et al.*, 2016). Thanks to these developments, researchers have begun to answer questions raised in 1993 when Com1 was first isolated, cloned, and sequenced (Hendrix *et al.*, 1990; Hendrix *et al.*, 1993). One of the earliest questions asked by Hendrix *et al.* upon sequencing *com1* and identifying it as a putative Dsb protein was: how is a putative outer membrane oxidoreductase related to pathogenesis? Subsequent efforts to answer that question came from an immunological perspective: identifying variations in *com1* sequences between isolates of *C. burnetii* from acute and chronic infections, use of Com1 as a diagnostic marker, and studying the efficacy of Com1 and Com1 peptide derivatives as Q fever vaccine candidates (Zhang *et al.*, 1997; Zhang *et al.*, 1998; Zhang and Samuel, 2003; Chen *et al.*, 2011; Peng *et al.*, 2012)

The recently developed ACCM-D culture methods, combined with bioinformatics, and new tools for genetically manipulating *C. burnetii* have allowed the pursuit of these answers from bioinformatic, biochemical, functional, and proteomic perspectives. Before addressing the question initially posed after *com1* was sequenced and identified to contain the CXXC motif characteristic of Dsb proteins, Com1 was characterized biochemically by purifying the protein and assessing Com1 oxidoreductase activity by the insulin disulfide reduction assay (Holmgren, 1979). Purified Com1 reduces insulin and at a rate that is characteristic of DsbC proteins (Zapun *et al.*, 1995). These results from the biochemical assays validated the findings from the bioinformatic analyses of this present study that also strongly suggest Com1 is a functional protein disulfide bond

isomerase protein, like DsbC proteins found in the periplasm of other Gram-negative bacteria.

While strategies of allelic replacement of *com1* with an in-frame deletion of the gene or replacement with a functionally inactive variant of the gene was beyond the scope of this initial study, use of the well characterized *dsb* mutant library of *E. coli* and pPDI detection system has been advantageous given the clarity of the results obtained (Ren and Bardwell, 2011). This ingeniously conceived system of testing protein disulfide isomerase has provided incontrovertible evidence that Com1 has protein disulfide isomerase activity as is evident by the ability of Com1 to rescue the growth phenotype of the Δ*dsbC E. coli* strain in the presence of inhibitory concentrations of ampicillin. In the absence of the native *E. coli* DsbC, the genetically modified β-lactamase on the pPDI vector can only be functional if DsbA forms the correct disulfide bonds initially or if a functional protein disulfide isomerase is able to rearrange incorrectly formed disulfide bonds to generate a functional β-lactamase that can permit the bacteria to grow in such high concentrations of ampicillin. This complementation strategy confirmed the protein disulfide isomerase activity of Com1. This information provided the foundation for further exploration about how Com1 functions in *C. burnetii* NMII through localization, determination of redox states, and identification of Com1 substrates.

Observers may note that a some differences existed between the assays performed with purified Com1 protein and those assaying Com1 function in the *E. coli* model system. In

particular the virtual absence of insulin reduction with purified P219T Com1 and the evidence that P219T Com1 has a low basal level of activity in protein disulfide isomerase activity in the pPDI detection experiments. While each of the assays measures different enzymatic activities, oxidoreductase activity vs. protein disulfide isomerase activity and each assay occur in widely different circumstances, *in vitro* vs. *in vivo*, it is worth exploring additional factors that may contribute to the differences observed. Primarily the *in vitro* assay with purified protein tested only the activity of the proteins in the absence of any other proteins, whereas the *in vivo* assay contained a functional DsbA protein. The presence of DsbA in the *in vivo* assay may have allowed for proper folding of a small percentage of β-lactamase proteins in the P219T Com1 strains. However, the strains with the empty vector control have a minute amount of bacterial growth. This may be attributable to growth deficiencies of the empty vector control strains where periplasmic proteins are unable to provide adequate functions for the bacteria to properly grow without a protein disulfide isomerase. Additional studies would be required to determine if the elimination of both DsbA and DsbC from these *E. coli* strains would provide results that align more completely with those observed in the *in vitro* study.

Determining the *in vivo* redox states of Com1 or any protein in *C. burnetii* a decade ago would have been a considerable undertaking if not logistically impractical with the cell culture-based method of cultivating *C. burnetii* strains. Making use of the ACCM-D culture method, the redox state of the recombinant 6x Histidine-tagged Com1 expressed in *C. burnetii* NMII has been determined, which revealed the typical redox states seen in

other Gram-negative bacteria (Joly and Swartz, 1997; Denocin *et al.*, 2013). Demonstration of the technique will allow for further exploration of the interactions between Com1 and putative Dsb proteins of *C. burnetii* to establish a complete picture of the disulfide bond creation and isomerization system. This is a critical future avenue of research that will reveal how formation and isomerization of disulfide bonds influence Q fever pathogenesis.

Since the initial study that sequenced the *com1* gene and revealed that sera from animals immunized with Com1 would not bind to boiled whole cell *C. burnetii* after proteinase K treatment, the outer membrane localization was a certainty (Hendrix *et al.*, 1993). However, monoclonal antibody generation to perform epitope mapping to determine what portion of Com1 was targeted by the antibody was never established. Without this information, there was no certain way to establish if a portion of Com1 is anchored in the outer membrane, or if the active site of Com1 is extracellular. Efforts to resolve this uncertainty from the early studies was made with immunogold labeling of the recombinant 6x Histidine-tagged Com1 protein expressed in *C. burnetii* NMII and visualization by transmission electron microscopy. The recombinant protein was engineered to have the histidine tag linked to Com1 downstream of the predicted signal sequence cleavage site (between positions 21 and 22 of Com1) to ensure that the tag would co-localize with Com1 after cleavage of the signal sequence. While preliminary trials staining the resin-embedded sections with different antibody dilutions have been unsuccessful at providing unambiguous localization details from NMII, the use of the same expression vector in *E. coli* to identify localization information from that system

was employed.

To address the question of how a Dsb protein is linked to virulence of *C. burnetii*, first posed by Hendrix *et al.*, when *com1* was first sequenced, the strategy pioneered by Kadokura *et al.* to capture substrates of DsbA as well as DsbC and Com1-like proteins was used by substituting the conserved proline opposite the CXXC active site with a threonine (Kadokura *et al.*, 2004; Jameson-Lee *et al.*, 2011). This amino acid substitution creates a permanent disulfide bond between the Dsb protein and the substrate. First pass analysis of this strategy with a recombinant 6x histidine-tagged Com1 P219T revealed key substrates of the T4BSS system, DotA and DotH. Additionally, putative substrates were identified linked to superoxide dismutase SodC and the potential virulence factor, Serine-type D-Ala-D-Ala carboxypeptidase. In addition to these obvious virulence-related proteins, the screen also identified seventeen uncharacterized proteins unique to *C. burnetii* strains. These results will stimulate new inquiries into the functions, if any, of these uncharacterized proteins in Q fever pathogenesis.

Unlike previous studies that made use of the substrate capture phenotype of the conserved proline-to-threonine substitution, this present study did not first separate proteins purified proteins with 2D-gel electrophoresis. The application of 2D-gel electrophoresis after elution of purified proteins from Ni-NTA resin had the advantage of visually selecting individual bands for sequencing based upon shifts in migration compared to the control (Kadokura *et al.*, 2004, Jameson-Lee *et al.*, 2011). This individual sequencing method had the advantage of minimizing non-specific and false-positive

results and identifying only protein hits with one or more cysteine residues. The approach of this present study to analyze the purified proteins directly with mass spectrometry allowed for more sensitive detection of protein hits at the cost of higher background of non-specific protein hits. To reliably exclude non-specific protein hits from the hits with interactions with Com1 requires three to five replicates for both the substrate capture and control and a statistical analysis of the replicates to validate protein hits that have a high degree of confidence to be linked to Com1 interactions. Limitations exist with this approach of directly sequencing purified protein, such as the technical challenges of growing and isolating proteins from 4 L of culture for six or more replicates each. Additionally, the use of trypsin, which cleaves lysine and arginine residues on the C-terminal side, has the potential to exclude proteins lacking those amino acid residues.

Based upon the results obtained for both the P219T Com1 substrate capture and the controls, likely artifacts can be identified for potential exclusion from the data sets. Primarily, protein hits with no cysteine residues found in the P219T Com1 substrate capture results are regarded as artifacts with likely affinity to Ni-NTA resin. Protein hits with high affinity for Ni-NTA resin are likely the most abundant artifacts present as highlighted by the co-chaperonin GroES (CBU_1719) and peptidyl-prolyl cis-trans isomerase, both of which have no cysteine residues, but for both the substrate capture and WT controls are among the top five hit frequencies. Homologs of these proteins have been identified in *E. coli* to have high affinity for Ni-NTA resin binding (Bolanos-Garcia and Davies, 2006).

When results of all replicates are completed, all data will be analyzed by pairwise t-tests to identify statistically significant hits in the P219T Com1 substrate group. It is expected that protein hits with high frequencies in both the substrate capture and WT control groups will likely be artifacts. Protein hits with one cysteines, like DotH/IcmK (CBU_1628) found in Table 2-2, have been known to have that cysteine residue involved in intermolecular disulfide bonds that would be expected in large molecular complexes like the T4BSS (Kadokura *et al.*, 2004, Jameson-Lee *et al.*, 2011). Proteomic analyses of the results with one or more cysteine residues will be analyzed with disulfide bond predictive software to build confidence in the validity of the hits. Analysis of each of the protein hits for the location of cysteine residues will then commence. This will ensure that protein hits with cysteine residues present only in signal sequences will be excluded as likely candidate substrates of Com1.

Using the above described processes to analyze the protein hits has identified likely substrates that include components of the T4BSS, like DotA, which was expected. Proteins of particular interest would be expected to have higher hit frequencies compared to the same proteins identified in the WT Com1 controls, as in the case of DotA. The higher hit frequencies of DotA in the P219T Com1 substrate capture samples strongly suggests that DotA protein abundance in this sample has been enriched through entrapment with Com1. Based on the results of previous studies, DotA is a structural component of the T4BSS in both *L. pneumophila* and *C. burnetii* (Roy *et al.*, 1998; Beare *et al.*, 2012). Preventing the formation of putative disulfide bonds of DotA

with the P219T substrate capture mutant would be expected to impair T4BSS formation and reduce virulence. It is noteworthy that protein hits containing one cysteine residue are also valid targets worth further exploration, depending on the accessibility of the cysteine residue, since proteins with one cysteine residues have been found to be essential for intermolecular disulfide bonds as might be expected to be required in a macromolecular complex like the T4BSS (Kadokura *et al.*, 2004, Jameson-Lee *et al.*, 2011). Ultimately, Com1 substrates would require validation through additional means including co-immunoprecipitation with antibodies specific for both Com1 and the substrate as well as AMS trapping of the potential substrates *in vivo* to confirm that the redox potential exists for disulfide bond formation.

This study is a first step into understanding disulfide bond formation in *C. burnetii* and the interactions required for the maintenance of the redox states of Dsb protein in the periplasm and inner membrane. The apparent lack of redundant Dsb protein homologs and orthologs present in the *C. burnetii* genome and the highly conserved sequences between the isolates suggest that Dsb proteins may be critical for bacterial growth and intracellular survival. Com1 substrates identified in this study, such as components of the essential T4BSS, certainly indicate that Com1 may be an essential protein. Ongoing studies testing the effects of Com1 P219T in cell line macrophage and mouse infections will soon begin to shed light into how irreversibly binding substrates in the periplasm can affect intracellular growth and virulence.

Experimental Procedures

Bacterial strains and culture methods

C. burnetii avirulent Nine Mile phase II (NMII) clone 4 RSA 439, was used for all studies involving the introduction of foreign genetic elements and genetic manipulation of the NMII strain genome. *C. burnetii* virulent Nine Mile phase I (NMI) clone 7 RSA493 sequence type: ST16, was used as the source for genetic material for recombinant protein studies (Glazunova *et al.*, 2005). Culturing of these strain utilized the liquid and solid defined acidified citrate cysteine media (ACCM-D) and cultured at 37°C in 5% CO_2 and 2.5% O_2 for 7 to 14 days (Sandoz *et al.*, 2016). Modifications to the ACCM-D (Appendix Table A-2) protocol included using frozen aliquots of the 4 mM $FeSO_4$, frozen immediately after preparing fresh and mixing well for each batch of ACCM-D prepared, frozen aliquots of 200 mM L-glutamine (Invitrogen) were also used for each batch of ACCM-D. Modifications to solid ACCM-D plating procedures included mixing 2X ACCM-D and 0.5% (wt/vol) UltraPure Agarose (Invitrogen) at a ratio of 1:1.08 and pouring plates of 12.5 mL the mixture into 100 mM Petri dishes without the use of an agarose overlay. The *E. coli* strain used for the Gateway® System (Invitrogen) cloning methods with pENTR vectors was TOP10™, genotype: F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G (Invitrogen). The *E. coli* strain used for propagating genes of interest and for generation of point mutants was ig™ 5a, genotype: Φ80 Δ(*lacZ*)M15 *fhuA2* Δ(*argF-lacZ*)U169 *phoA glnV44 gyrA96 recA1 relA1 endA1 thi-1hsdR17* (Intact Genomics, Inc). The *E. coli* strain used for expression of recombinant proteins was BL21-AI™, genotype: F- *ompT hsdS*$_B$

(r_B-m_B-) *gal dcm araB*::*T7RNAP-tetA* (Invitrogen). The *E. coli* strain used for construction of vectors using In-Fusion® HD Cloning Kit was Stellar™, genotype: F-, *endA1*, *supE44*, *thi-1*, *recA1*, *relA1*, *gyrA96*, *phoA*, Φ80d *lacZΔ* M15, Δ(*lacZYA-argF*) U169, Δ(*mrr-hsdRMS-mcr*BC), Δ*mcrA*, λ- (Takara Bio USA, Inc.).

Primers and plasmids

All vectors for auxotroph construction were generously provided by Dr. Paul Beare at Rocky Mountain Laboratories. Recombinant *dsb* genes were initially cloned into the pENTR™/SD/D-TOPO® Cloning Kit (Invitrogen) following manufacturer's protocols with pENTRCom1P219T-For and pENTRCom1P219T-Rev primers and sequence verified with M13 Foward and Reverse primers (Table 2-3). Transfer of the pENTR *com1* genes was achieved using the Gateway® pDEST17 vector system following manufacturer's protocols and sequences were verified with T7 promoter and terminator primers (Invitrogen). Removal of the pDEST 6xHis tag upstream of the *com1* signal sequence and insertion of the 6xHis tag between the signal sequence and *com1* sequence was achieved with a three rounds of round-the-horn site-directed mutagenesis with 20200302a_pDEST-veR6H19_F and 20200302b_pDEST-veR6H19_R, 20200302c_pDEST-1H-6-10aaLnkDD19_F and 20200302d_pDEST-1H-6-10aaLnkDD19__R, and 20200302e_pDEST-6H-10aaLnkDD19_F and 20200302f_pDEST-6H-10aaLnkDD19_R and sequence verified with T7 sequencing primers. Auxotrophic vector pJBProBA-Amp was modified pJBProBA-Kan with 20190321a_pJBProBA_base4245_For, 20190321b_pJBProBA_base3096_Rev primers, and cloning in *kan* with 20190321c_KanR_gene_For and 20190321d_KanR_gene_Rev with the In-Fusion® HD

Cloning Kit (Takara Bio USA, Inc.) following manufacturer's protocol and utilizing the manufacturer's oligo synthesis generation online tools. Recombinant *dsb* genes were transferred from pENTR vectors into modified auxotrophic vectors by sequence- and ligation-independent cloning (SLIC) methods with the 5' pJB-Pro-pDEST_Universal_For and 3' pJB-Pro-pDEST_Universal_Rev primers and sequences were verified with 20181002a-pJBpTac-seq-For and 20181002b-pJB2xHA-seq-Rev primers. Point mutations of recombinant proteins were generated in the pENTR backbone by round-the-horn site-directed mutagenesis with the 5'-3'Com1P219T_forward and 3'-5'Com1P219T_reverse primers (Moore and Prevelige, 2002; Openwetware.org, 2018). Plasmid isolation for transformation into *C. burnetii* Nine Mile phase II strain, RSA439, was achieved with Maxi Plasmid Isolation Kits (Qiagen) following the manufacturer's protocol. Final plasmid elution in sterile nuclease-free H_2O was concentrated on Amicon® Ultra 3K MWCO Centrifugal Filters (EMD Millipore) to approximately 4 μg/μL prior to transformation.)

Bioinformatic, phylogenic, and sequence alignment analyses

The conserved Dsb protein active-site, CXXC motif was used to identify Dsb proteins in *C. burnetii* and identified amino acid sequences in all published *C. burnetii* genomes of four distinctly different Dsb proteins. Clustal Omega was used for sequence alignment of *C. burnetii* Dsb proteins with *E. coli* and *L. pneumophila* Dsb proteins. Dendrograms from sequence alignments were generated with Dendroscope version 3.5.9 (Huson and Scornavacca, 2012)

Table 2-3. List of primers.

Primer Designation	Primer Sequence 5' to 3'
M13 Forward	GTAAAACGACGGCCAG
M13 Reverse	CAGGAAACAGCTATGAC
T7 promoter	GCATAATACGACTCACTATAG
T7 terminator	GCTAGTTATTGCTCAGCGG
5'-3'Com1P219T_forward_primer	ACGACGTTCGTCATTGGTAATAAAGCGTTAACC
3'-5'Com1P219T_reverse_primer	GGTGCCTGCTAGCTGTAACGATTGAG
pENTRCom1P219T-For	ACGACGTTCGTCATTGGTAATAAAGCGTTAACC
pENTRCom1P219T-Rev	GGTGCCTGCTAGCTGTAACG
20181002a-pJBpTac-seq-For	CGACATCATAACGGTTCTGGC
20181002b-pJB2xHA-seq-Rev	GCTACAAATGGGTGATAAGAGGG
5' pJB-Pro-pDEST_Universal_For	CCTTCATGAAGGAGGCTGCAGATGTCGTACTACCATCACCATCACCATC
3' pJB-Pro-pDEST_Universal_Rev	CATCGTATGGGTACATCTGCAGCTCGAATCAACCACTTTGTACAAGAAAGCTG
20190321a_pJBProBA_base4245_For	ACCAAGTTTACTCATATATACTTT
20190321b_pJBProBA_base3096_Rev	GACAAACAACAGATAAAACGAAA
20190321c_KanR_gene_For	TATCTGTTGTTTGTCTCAAAATCTCTGATGTTACATTGCA
20190321d_KanR_gene_Rev	ATGAGTAAACTTGGTTCCCGTCAAGTCAGCGTA
20200212a_pJBProBA_5'tag_F	ATGAAGAACCGTTTGACTGCACTATTTTTAGCC
20200212b_pJBProBA_5'tag_R	TGCTTTTTTGTACAAACTTGTTGATTCGAGGTG
20200302a_pDEST-veR6H19_F	ATGAAGAACCGTTTGACTGCACTATTTTTAGCC
20200302b_pDEST-veR6H19_R	ATGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGAAACCG
20200302c_pDEST-1H-6-10aaLnkDD19_F	TTGTACAAAAAAGCAgccccctctcaattcag
20200302d_pDEST-1H-6-10aaLnkDD19_R	ATGGTAGTATGGTGCggctatcgccacgc
20200302e_pDEST-6H-10aaLnkDD19_F	CTCGAATCAACAAGTTTGTACAAAAAAGCAgccc
20200302f_pDEST-6H-10aaLnkDD19_R	GTGATGGTGATGGTGATGGTAGTATGGTGC

Generation, isolation, and purification of recombinant proteins

Recombinant proteins were generated from isolated genomic DNA of the virulent Nine Mile phase I clone 7 RSA 493, of *C. burnetii* for all studies to ensure that the results are the most relevant to proteins from strains that cause pathology in humans and livestock. Recombinant proteins for biochemical assays were generated from expression vectors in *E. coli* strain BL21-AI™ and induced with 0.5 mM IPTG (Fisher Scientific or Gold Biotechnologies, Inc.) for 3 to 4 hours at 37°C, shaking at 200 rpm. Induced cultures were harvested by centrifugation at 3700 x *g* for 30 min at 4°C, pellets were resuspended in chilled Ni-NTA Buffer A (30 mM KPO_4, 300 mM NaCl in ddH_2O) and 1 mM PMSF (Millipore Sigma) and were stored at -80°C or immediately sonicated to disrupt the bacteria and release the recombinant proteins. Lysates loaded onto nickel agarose bead columns to isolate the 6x Histidine-tagged recombinant proteins from the lysates. Proteins were eluted using set gradients of Ni-NTA Buffer A, supplemented with Ni-NTA Buffer B (30 mM KPO_4, 300 mM NaCl, 500 mM imidazole in ddH_2O) for elutions of protein at 25 mM, 50 mM, 100 mM, 150 mM, 250 mM imidazole. Fractions of each elution are visualized on by SDS-PAGE on 15% acrylamide gels to determine which fractions to concentrate by Vivaspin 20 5,000 MWCO concentration tubes (GE Healthcare, USA). Selected fractions are concentrated in Vivaspin tubes according to the manufacturer's protocol and the imidazole was removed by continual rinses and concentrations in phosphate buffered saline (PBS) or 0.1 M sodium phosphate buffer with 2 mM EDTA, pH 7 for insulin disulfide reduction assays. Recombinant proteins were supplemented with 20% glycerol prior to storage at -80°C.

Transformation of C. burnetii Nine Mile phase II strain

Preparation of fresh electrocompetent *C. burnetii* NMII followed established protocols with ACCM-D (Beare *et al.*, 2014). Successful transformation of electrocompetent *C. burnetii* Nine Mile phase II strain RSA439 was achieved using approximately 8 μg of concentrated plasmid stocks (see *Primers and plasmids* in Materials and Methods) per 50 μL of freshly prepared electrocompetent *C. burnetii* Nine Mile phase II strain RSA439 harvested at mid-logarithmic growth (approximately 6 to 7 days). The mixture of 50 μL fresh electrocompetent cells and 8 μg plasmid DNA was transferred to a chilled, sterile 1 mm gap electroporation cuvette (Bio-Rad). The mixture of bacteria and plasmid DNA was electroporated at 18 kV/cm, 25 μF, and 500 W, as has been previously published (Beare *et al.*, 2009). Immediately following transformation, 0.95 mL of RPMI 1640 + Glutamax supplemented with heat inactivated 1% Fetal Bovine Serum (Gibco) was transferred to the cuvette and vigorously pipetted and the contents of the cuvette were transferred to a sterile 1.5 mL microcentrifuge tube until the transformed bacteria could be added to culture. For bacteria transformed with plasmids containing the *proBA* selection cassette, 0.2 mL of the transformed culture in RPMI 1640 + Glutamax, supplemented with 1% FBS was added to 3 mL freshly prepared ACCM-D without proline in a T-25 filter cap flask. All cultures were incubated at 37°C in 5% CO_2 and 2.5% O_2 for 7 days and the remainder of the transformed cultures were supplemented with 88 mL of DMSO for a final concentration of 10% DMSO and store at -80°C.

Insulin disulfide reduction assay

Following the methods outlined by Holmgren (1979), the insulin disulfide reduction assay utilizes the precipitation properties of insulin when its disulfide bonds are reduced by a reductase in the presence of DTT. At pH 7.0 1 M insulin, in Tris-Base, was completely dissolved in solution. When reduced by DTT or a reductase the two interchain disulfide bonds of the A and B chains of insulin will cause cleavage of the chains, resulting in precipitation of the cleaved A and B chains of insulin. As a non-enzymatic control, insulin was treated by DTT alone in the absence of Com1. This induced a 10^4 reduction in the rate constant compared to one driven by a reductase. Using a 96-well optical flat-bottom ELISA plate, a constant volume of insulin (150 μM) and DTT (0.33 mM) in 100 mM sodium phosphate buffer was plated 200 μL per well as described by Kpadeh *et al.*, 2013. Dilutions of Com1 (0 – 20 μM) initiated the reaction, measured by absorbance at 650 nm. This established the Com1 enzyme activity compared to the non-enzymatic DTT + insulin control.

Localization of Com1 using transmission electron microscopy

To determine the localization of recombinant 6x-Histidine tagged Com1 in *C. burnetii*, 50 mL cultures of transformed NMII were grown for 7 days and induced with filter sterilized 0.5 mM IPTG (Gold Biotechnology, USA) for 16 hours. Then, 6 mL of cultures of empty vector control and WT Com1 were centrifuged at 16,000 x *g* for 15 minutes and supernatants were carefully aspirated and cell pellets resuspended in 1 mL of primary immuno-fixative provided by the University of Missouri Electron Microscopy Core: 100 mM sodium cacodylate, 2% (vol/vol) glutaraldehyde, 2% (vol/vol)

paraformaldehyde and incubated at room temperature for 30 minutes and the fixed cultures were centrifuged again at 16,000 x *g* for 15 minutes and carefully aspirated and resuspended in 0.1 mL of immuno-fixative and submitted to the MU Electron Microscopy Core for processing. Briefly, fixed cultures were embedded in London Resin (LR) White (Electron Microscopy Sciences, USA) and sectioned. Immunostaining followed previously published procedures (Fernandez *et al.*, 1996; Skepper and Powell, 2008). Briefly, sections were first blocked in 1X phosphate buffered saline gelatin (PBSG) containing 1% (wt/vol) gelatin (cold water fish) (Millapore Sigma), 0.001% Tween 20 (Amresco), and 0.001% Triton X-100 (Amresco) for 10 minutes by placing the grids on drops of the solution. Following blocking, sections sections were treated with primary murine-derived Anti-6xHis-Tag antibody (1:20) (Invitrogen) diluted in 1X PBSG overnight. Treated sections were subsequently washed on ten 0.1 mL drops of 1X PBS for two minutes on each drop. Sections were then treated with secondary 10 nm diameter gold particles conjugated with goat-derived anti-mouse antibody (1:20) (Millipore Sigma) for 2 hours. Sections were then rinsed in ddH2O for 30 seconds and submitted again to the MU Electron Microscopy Core for analysis by transmission electron microscopy (JEOL JEM 1400 transmission electron microscope – JEOL, Peabody, MA) at 80 kV on a Gatan Rio CMOS camera (Gatan, Inc. Pleasanton, CA).

Dsb protein complementation study

Complementation of *E. coli dsbC* mutant with pJB-ProBA-Kan vectors encoding WT Com1, P219T Com1, or empty vector was performed as outlined by Ren and Bardwell in 2011 and Kpadeh *et al.* in 2015. Briefly, RGP *dsb* mutant strains carrying pPDI detector

plasmid were transformed with each of the three pJB-ProBA-Kan plasmids (empty, WT Com1, or P219T Com1) and selected, screened, and clonal isolates propagated on LB Miller agar containing 100 μg/mL Ampicillin and 50 μg/mL Kanamycin. Overnight cultures grown in LB Miller broth containing 100 μg/mL Ampicillin and 50 μg/mL Kanamycin were diluted 1:100 in fresh broth with selection and with or without induction of Com1 with 1 mM IPTG and cultures were incubated at 37°C with shaking at 200 rpm. The culture OD_{600} was monitored and once cultures reached OD_{600} = 0.6, the cultures were placed immediately on ice and then diluted in 150 mM NaCl and spotted on LB Miller agar plates supplemented with 1, 2, or 3 g/L of ampicillin.

Identification of Com1 substrates by mass spectrometry

Cultures of 1X ACCM-D without proline, pH 4.75 transformed with pJBProBA-Kan-signal-sequence-6xHis-Com1P219T incubated at 37°C in 5% CO_2 and 2.5% O_2 for 7 days were expanded until passage 6 when culture densities were sufficient to expand to 4 L of 1X ACCM-D without proline, pH 4.75, in twenty 300 cm^2 cell culture flasks with 200 mL of 1X ACCM-D without proline, pH 4.75 each and incubated at 37°C in 5% CO_2 and 2.5% O_2 for 6 days. At 6 days, cultures were induced with 0.5 mM IPTG (Gold Biotechnologies, USA) for 16 hours prior to harvest. Harvest of proteins from periplasmic and outer membrane of *C. burnetii* NMII was achieved using the Tris-sucrose-EDTA (TSE) method (Quan *et al.*, 2013). Briefly, cultures were centrifuged in chilled 750 mL centrifuge bottles (Thermo Scientific, USA) at 3,000 x *g* for 20 minutes at 4°C. Supernatants are carefully decanted and remaining culture media removed by

aspiration. Each pellet was resuspended in 7.5 mL of 40 mL prepared TSE buffer (200 mM Tris-Base, pH 8.0, 500 mM sucrose, 1 mM EDTA with 0.4 mL of Halt Protease and Phosphatase Inhibitor (Pierce, USA) and incubated on ice for 30 minutes. Resuspensions were transferred to chilled 50 mL conical centrifuge tubes and centrifuged at 16,000 x *g* for 30 minutes at 4°C. Supernatants containing the periplasmic and envelope extract were transferred to clean 50 mL tubes and treated with ice cold trichloroacetic acid, final concentration 10% and incubated on ice for 30 minutes. Precipitated protein pellets were washed twice in 100% acetone by centrifugation at 16,000 x *g* for 15 minutes at 4°C. Washed protein pellets were dried and resuspended in 10 mL of 100 mM Tris pH 8.0 containing 0.5% (wt/vol) SDS and 100 mM iodoacetamide. Resuspended pellets were further diluted four-fold in 50 mM Tris pH 8.0 with 300 mM NaCl. Removal of insoluble material was achieved by centrifugation at 10,000 x *g* for 20 minutes at 4°C and supernatants were transferred to clean 50 mL tubes. Final protein supernatants were loaded onto 1 mL of Ni-NTA resin (Gold Biotechnology, USA) equilibrated with 50 mM Tris pH 8.0 with 300 mM NaCl and 0.1% SDS (wt/vol). Proteins were eluted using set gradients of 50 mM Tris pH 8.0 with 300 mM NaCl and 0.1% SDS (wt/vol), supplemented with imidazole for elutions of protein at 25 mM, 50 mM, 100 mM, 150 mM, 250 mM imidazole. Typical elution is shown in the appendix (Appendix Figure A-1) Fractions of each elution are visualized on by SDS-PAGE on 15% acrylamide gels to determine which fractions to concentrate by Vivaspin 20 5,000 MWCO concentration tubes (GE Healthcare, USA). Selected fractions are concentrated in Vivaspin tubes according to the manufacturer's protocol and the imidazole was removed by continual rinses to replace the buffer with 30 mM KPO4 pH 8.0. Following buffer replacement,

samples were treated with 3 volumes of ice cold acetone overnight at -20°C. The precipitated protein was isolated by centrifugation at 20,000 x *g* for 5 minutes at 4°C and washed three times with 80% acetone. Pellets were then transferred to the Proteomics Center where they were washed one time with 80% acetone in water and then resuspended in 20 µL of urea buffer (6M urea, 2M thiourea, 100mM ammonium bicarbonate, pH 8.0). Resuspended proteins were digested with 0.2 µg of LysC overnight and then 0.2 µg of trypsin for 8 hours according to SOPs. Peptides were then desalted and concentrated using C18 tips (Cat# 87784, Lot# TG271472) according to the manufacturer's protocol (Pierce, USA).

Peptides were analyzed by mass spectrometry as follows: a 1 µL injection was made directly onto a 20 cm long x 75 µm inner diameter pulled-needle analytical column packed with Waters BEH-C18, 1.7 µm reversed phase resin. Peptides were separated and eluted from the analytical column with a gradient of acetonitrile at 300 nL/minute. The Bruker nanoElute system is attached to a Bruker timsTOF-PRO mass spectrometer via a Bruker CaptiveSpray source.

LC gradient conditions: Initial conditions were 3%B (A: 0.1% formic acid in water, B: 99.9% acetonitrile, 0.1% formic acid), followed by 25 minute gradient to 17%B, 17-25%B over 25 minutes, 25-37%B over 20 minutes, 37-80%B over 2 minutes, (oscillating column wash: 2 minutes to 40%B/80%B/40%B/80%B, hold at 80%B for 2 minutes, and then ramp back to (2 minutes) and hold at (6 minutes) initial conditions. Total run time was 90min.

MS data were collected in positive-ion data-dependent PASEF mode over an m/z range of 100 to 1700, last calibration date: 03/25/2022. PASEF and TIMS were set to "on". One

MS and ten PASEF frames were acquired per cycle of 1.1sec (~1MS and 120 MS/MS). Target MS intensity for MS was set at 10,000 counts/second with a minimum threshold of 250 counts/second. A charge-state-based rolling collision energy table was used from 76-123% of 42.0 eV. An active exclusion/reconsider precursor method with release after 0.4 minutes was used. If the precursor (within mass width error of 0.015 m/z) was >4X signal intensity in subsequent scans, a second MSMS spectrum was collected. Isolation width was set to 2 m/z (<700m/z) or 3 (800-1500 m/z).

Database searches (protein identification)

The acquired data were submitted to the PEAKS Studio search engine for protein identifications (Zhang *et al.*, 2012). The Uniprot *C. burnetii* database was used (1812 entries; last update 1/17/22) with an automated decoy database search for calculation of false discovery rate. Data were searched with "Trypsin" as enzyme; oxidized methionine and acetylation as variable mods; 20 ppm mass tolerance on precursor ions, 0.1 Da on fragment ions. Search results files were export from PEAKS Studio with FDR set at 1% and further filtered using excel by all combined protein ≥1 peptide per protein and ≥2 hit frequencies per sample.

Acknowledgments

The authors would like to thank Dr. James Bardwell for the generous donation of the *E. coli dsb* mutant strains used in this study. Special thanks go to Dr. Paul Beare and Dr. Kelsi Sandoz for sharing their time and advice and auxotrophic cloning vectors and

ACCM-D recipes that enabled all the *C. burnetii* culturing and cloning experiments in this study. NPO was supported by the University of Missouri Gus T. Ridgel Fellowship and Life Sciences Fellowship, NIH NIGMS IMSD training grant, and NIH T32 training grant in addition to funds from the College of Veterinary Medicine's Veterinary Pathobiology department.

Chapter 3

Antibiotic Susceptibility Factors in *Coxiella burnetii*

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Summary

The factors of *Coxiella burnetii*, causative agent of Q fever, that contribute to antibiotic susceptibility remain poorly characterized. This gap in knowledge of how bacterial factors contribute to antibiotic susceptibility has prevented advancements in treatment options for patients afflicted with Q fever beyond a dual course of doxycycline and hydroxychloroquine. This antibiotic therapy is not effective for all patients and reemergence of infections after treatment suggests there are unknown factors contributing to this resistance phenotype. Previous studies have not been able to determine if the resilience to antibiotic treatment of human Q fever cases is attributed to bacterial factors, the acidic intracellular environment of the *Coxiella* containing vacuole (CCV), or host factors. Identifying the presence and function of antibiotic susceptibility factors provides useful insight towards informed treatment regimens. Bioinformatic identification of putative antibiotic susceptibility genes was implemented to compare sequences and structures between strains associated with acute and chronic infections. In this study, using the defined acidified citrate cysteine medium (ACCM-D) (Sandoz *et al.*, 2016) developed for axenic culturing of *C. burnetii*, the effects of the acidic and microaerophilic environment of the *Coxiella*-containing vacuole (CCV) on antibiotic efficacy are highlighted.

Introduction

Given the threat posed to human health by air-borne bioterrorism agents like the obligate intracellular bacterial pathogen *C. burnetii*, it is critical to have effective therapeutics to treat those infected. Infection of humans with fewer than 10 bacteria of

C. burnetii can result in the disease Q fever that presents initially as a febrile illness that if untreated can develop into life threatening complications like atypical pneumonia, hepatitis, and endocarditis (Raoult *et al.*, 1990b; Maurin and Raoult, 1999; Raoult *et al.*, 2005). A recent outbreak in the Netherlands, beginning in 2007 and continuing unabated through 2010, resulted in over 4,000 individuals diagnosed with Q fever. This Q fever outbreak event emphasizes the importance of controlling this bacterial pathogen that commonly infects ruminants used in the livestock industry (Wouda and Dercksen, 2007; Enserink, 2010; van den Wijngaard *et al.*, 2011; van der Hoek *et al.*, 2012). This latest outbreak and data from previous studies shows that the communities surrounding livestock facilities are also at risk for infection from this air-borne pathogen (Tissot-Dupont *et al.*, 2004; van der Hoek *et al.*, 2012). In addition to being transmitted easily by aerosol, this pathogen develops a metabolically inert morphology called the small cell variant (SCV) enabling it to survive outside the host until inhaled or introduced subcutaneously. This SCV morphology is highly resistant to environmental stresses such as oxidation, ultra violet light, and desiccation (Babudieri, 1959; McCaul *et al.*, 1981). The ability of this bacterium to persist in adverse conditions is also seen in human infections, where chronic infections are treated with daily doses of antibiotic for up to 4 years (Angelakis and Raoult, 2010). The factors underlying this astounding persistence, despite a treatment combination of hydroxychloroquine and doxycycline (a tetracycline class antibiotic) has largely been unexplored, in large part because it has only been possible to culture the bacterium in cell lines and chicken eggs (Raoult *et al.*, 1990a). Recently developed axenic culture media has enabled pure cultures of the bacteria to be grown in both liquid and on solidified media, permitting,

for the first time, detailed genetic studies of this challenging pathogen. There is a need to improve patient outcomes and treatment by elucidating the molecular mechanisms responsible for persistent chronic Q fever infections. The majority of current studies in this field are focused on a single avirulent strain, Nine Mile phase II, that can be manipulated under Biosafety Level 2 (BSL-2) conditions. This focus has ignored the importance of the clinically relevant virulent strains. Bioinformatic analysis of published genomes of virulent strains isolated from acute and chronic infections allows for the findings of this work to have some clinical relevance. This study seeks to explore the factors involved in antibiotic susceptibility and resistance to establish key targets involved in enabling the persistence of chronic infections. The hypothesis explored here is that virulent strains isolated from chronic infections have acquired alleles associated with antibiotic resistance. This work will provide much needed details on the mechanisms of antibiotic resistance of this difficult to treat pathogen and in doing so, identify novel targets to improve the efficacy of treatment of individuals infected with Q fever.

The majority of antibiotic susceptibility studies of virulent *C. burnetii* strains occurred years before the development of the cell free growth medium, ACCM-D, and have been unable to distinguish between the host factors and the pathogen factors involved in antibiotic susceptibility (Yeaman *et al.*, 1987; Raoult *et al.*, 1989; Raoult *et al.*, 1990; Yeaman and Baca, 1990; Raoult *et al.*, 1991). Some of these early studied identified that the host contributed to factors that affected *C. burnetii* susceptibility to antibiotics. One such study tested the antibiotic treatment of the *C. burnetii* strain Q217 isolated from a

patient heart valve after several months of doxycycline and rifampin treatment, yet the strain was susceptible to those antibiotics when cultivated in cell culture (Yeaman and Baca, 1990). This group sought to further explore how the host impacts antibiotic treatment with tritium-labeled antibiotics where they found tritium-labeled tetracycline predominantly accumulated within the L929 cells and only a fraction of the tritium labeled-tetracycline was detected in *C. burnetii* isolated from those cells (Yeaman and Baca, 1991). To add to the complexity of factors involved, tritium labeled-tetracycline accumulation in the host cells occurred only when infected by *C. burnetii* strain NMI and not other strains and accumulation was not observed in other labeled antibiotics. These studies and others repeated found evidence that *C. burnetii* alters how permissible the host is to antimicrobial agents and alters the susceptibility of the pathogen to antibiotic exposure (Yeaman *et al.*, 1987; Yeaman *et al.*, 1989; Yeaman and Baca, 1990; Levy *et al.*, 1991; Raoult *et al.*, 1991a; Raoult *et al.*, 1991b; Raoult, 1993; Maurin and Raoult, 1999; Raoult *et al.*, 2002). However, in order to understand the nature of this pathogen's persistence to antibiotic treatment, it is critical to distinguish between the host- and pathogen-associated factors. Separating host- and pathogen-associated factors is made possible with the ACCM-D medium.

One thorough study of the bacterial outer membrane and envelope proteins did not find any putative antibiotic susceptibility proteins predicted to localize to the outer membrane or envelope, but did identify three genes annotated as predicted efflux proteins: CBU_0754, CBU_1094, and CBU_1811 (Flores-Ramirez *et al.*, 2014). However, Flores-Ramirez *et al.* found none of those three putative efflux transporter proteins in

the results from LC-MS/MS analysis of extracts from the outer membrane and periplasm. Though interestingly, Flores-Ramirez *et al.* did find a putative tetracycline resistance gene product that was not predicted to be present in the outer membrane, CBU_0235, which is a predicted elongation factor G protein with potential involvement in ribosomal protection. A recent study took the crucial first step of applying proteomic analysis to *C. burnetii* grown in ACCM-D to understand how the bacterium responds at a protein level to doxycycline treatment and identified increases in proteins related to protein translation and folding, which include a predicted elongation factor that had increased protein abundance when treated with doxycycline (Zuñiga-Navarrete *et al.*, 2019). Tetracycline class antibiotic compounds like doxycycline are one of the few effective treatments for Q fever (Angelakis and Raoult, 2010).

There is an urgent need for a comprehensive re-evaluation of the factors involved in antibiotic susceptibility of *C. burnetii* that utilizes bioinformatic analyses to identify putative gene products that may contribute to antibiotic susceptibility. Additionally, new methodology is needed to standardize antibiotic susceptibility studies with those methods approved by Clinical and Laboratory Standards Institute (CLSI) that can now be performed with the recent liquid and solidified axenic medium (CLSI, 2012; Sandoz *et al.*, 2016). These approaches combined with standard cloning methods can help characterize gene products of *C. burnetii* to better understand what role they have in contributing to the resilience of *C. burnetii* to antimicrobial agents.

In this study the genetic components derived from the DNA sequences of *C. burnetii*

strains were explored as they relate to putative antibacterial susceptibility proteins. The relationships of these genetic components were explored among *C. burnetii* strains isolated from invertebrates, livestock, and patients treated for Q fever. Then the effects that the acidic environment, in which *C. burnetii* grows, has on antibiotics were explored in the axenic medium with the well characterized *E. coli* bacterial system. Then new culture and plating methods were explored that allow for more traditional antimicrobial testing methods to be applied to *C. burnetii* strains grown in axenic media, providing novel insights into differences among isolates.

For the purposes of this study, antibiotic susceptibility is defined as it relates to genes and the proteins encoded by those genes as a spectrum where alleles can confer no resistance, intermediate resistance, or full resistance to specific antibiotics. The antibiotics used in this study have different targets and mechanisms of actions and the presence or absence of genes and the proteins encoded by those genes affects the interactions of antibiotics with the bacterium and therefore how susceptible the bacterium is to antibiotic treatment in the conditions assayed in this study.

Results

Identification of putative antibiotic susceptibility genes in C. burnetii strains

An exhaustive and completely comprehensive analysis of all potential genetic factors that may contribute to antibiotic susceptibility in *C. burnetii* is beyond the scope of this limited study. In an attempt to establish links between protein function and response to specific classes antibiotics, genes annotated as multidrug efflux pumps were excluded

and focus was made on gene annotations with well established direct links to unique classes of antibiotics. The *C. burnetii* NMI strain reference genome was used as the primary source for identifying genes with annotations associated with antibiotic susceptibility. Review of the gene annotations in the NMI genome identified eight potential genes of interest, protein sequences from each of the eight were cross-referenced for homology to characterized proteins in other bacteria. Subsequently, published data on proteomic analyses of *C. burnetii* were reviewed to identify which of the proteins of interest have been confirmed to be present in two or more independent proteomic analyses. Confirmation of protein expression was used to continue bioinformatic comparisons for the rest of this study. Of the initial eight cross-referenced gene annotations, only six were confirmed to express proteins from two or more proteomic studies and one was identified as a multi-drug efflux pump and was excluded from further analysis (Table 3-1). The table reveals confirmed expression of a putative DNA gyrase subunit A protein (CBU0524), β-lactamase serine hydrolase (CBU0807), streptogramin A acetyltransferase-like protein (CBU0832), TypA GTP-binding protein (CBU0884), MefA-like macrolide efflux protein (CBU1896). It is noteworthy to mention that the annotated *ksgA* (CBU1982) thought to encode a methyltransferase with alleles that can confer susceptibility or resistance to aminoglycosides was not identified in any proteomic studies (Duffin and Seifert, 2009; Ochi *et al.*, 2009). Additionally, a gene with homology to the *E. coli qacE* gene, which encodes a protein known to promote resistance a class of antimicrobial detergents and disinfects known as quaternary ammonium compounds was identified only in the genomes of *C. burnetii* Dugway 5J108-111 and two Dugway substrains, *qacE*

Table 3-1. Identification of genes with putative roles in response to antimicrobial agents. Locus tags for each strain are listed for antimicrobial class if present. The symbol, *, indicates locus ID is that of Dugway 5J108-111, all others refer to strain RSA493 NMI. References for studies which identified the proteins are Skultety *et al.*, 2011 (a); Dresler *et al.*, 2019 (b); Schmoock *et al.*, 2019 (c).

Gene Annotations	NMI locus ID	Protein Confirmed	Relevant Antimicrobial Class
gyrA DNA gyrase subunit A	CBU0523	b, c	Quinolones
β-lactamase family protein	CBU0807	b, c	β-lactams
Acetyltransferase	CBU0832	b, c	Streptogramins
TypA/BipA GTP-binding protein	CBU0884	a, b, c	Tetracyclines
Bcr family multidrug resistance transporter	CBU0959	b, c	Chloramphenicols & other antibiotic classes
Macrolide-efflux protein	CBU1896	b, c	Macrolides
ksgA dimethyladenosine transferase	CBU1982	No confirmation	Aminoglycosides
Multidrug efflux SMR transporter	CBUD0802*	No confirmation	Quaternary ammonium compounds

(CBUD0802),. Given the absence of proteomic studies on this strain of *C. burnetii*, this was also excluded from further analysis.

Having identified five putative genes known to encode proteins in the NMI reference strain, homologs for the protein sequences encoded by these genes were searched for in the annotations of nine strains of *C. burnetii*, two of which (Q229 and Q321) do not have fully assembled genomes, but have searchable protein tables (Table 3-2). This initial screen of eight fully sequenced and published genomes of *C. burnetii* and the protein tables for Q229 and Q321 revealed the presence of all five proteins associated with antibiotic susceptibility to five different classes of antimicrobials (β-lactams, macrolides, quinolones, streptogramins, and tetracyclines).

Early efforts to differentiate *C. burnetii* strains by serological methods failed to provide any meaningful results of this Gram-negative obligate intracellular bacterial pathogen and created a perception of a high degree of conservation among the various isolates (Peacock *et al.*, 1983). However, bioinformatic analysis of the published genomes reveals a variety of differences among the strains, especially as it relates to the presence, absence and sequence identity of putative antibiotic susceptibility related genes. The genomes of ten isolates of *C. burnetii* (CbuG-Q212, CbuK-Q154, Dugway 5J108-111, Namibia, Priscilla MSU Q177, Q321, RSA 331, RSA 493 Nine Mile I) were surveyed for the presence of genes involved in putative antibacterial susceptibility among five classes of antimicrobial agents (Table 3-2). This initial survey reveals that all ten isolates (CbuG-Q212, CbuK-Q154, Dugway 5J108-111, Namibia, Priscilla MSU 'Goat'

Table 3-2. Bioinformatic identification of genes from eight *C. burnetii* strains with putative relation to eight classes of antimicrobial agents. Locus tags for each strain are listed for antimicrobial class if present. The genome for Q321 is currently in unassembled contigs and locus tags are not present for all proteins, if not available, then N/A is used. If protein is not encoded by the genome, then not present (N/P) is used.

Coxiella Strain Designation	β-lactams	Macrolides	Quinolones	Streptogramins	Tetracyclines
Dugway 5J108-111	CBUD_0873	CBUD_0127	CBUD_1539	CBUD_0897	CBUD_0948
G Q212	CbuG_1193	CbuG_0271	CbuG_1470	CbuG_1169	CbuG_1118
K Q154	CbuK_0676	CbuK_0196	CbuK_1312	CbuK_0700	CbuK_0749
MSU 'Goat' Q177	A35_03355	A35_00970	A35_06705	A35_03470	A35_03725
Namibia	CBNA_0638	CBNA_0171	CBNA_1314	CBNA_0662	CBNA_0713
Q229*	CbuQ229_RS06040	CbuQ229_RS01375	CbuQ229_RS07460	CbuQ229_RS05915	CbuQ229_RS05655
Q321*	COXBURSA334_RS05165	COXBURSA334_RS06255	COXBURSA334_RS09705	COXBURSA334_RS06255	COXBURSA334_RS02205
RSA331 Henzerling	COXBURSA331_A1144	COXBURSA331_A2100	COXBURSA331_A0638	COXBURSA331_A1118	COXBURSA331_A1063
RSA493 Nine Mile I	CBU_0807	CBU_1896	CBU0524	CBU_0832	CBU_0884
Scurry Q217	AYM17_05805	AYM17_01290	AYM17_07205	AYM17_05685	AYM17_05425

Q177, Q229, Q321, RSA331, RSA493 NMI, Scurry Q217) have genes that are putatively related to antibiotic susceptibility. This initial survey may suggest a high degree of conservation among the isolates, a much more interesting picture emerges when the proteins encoded genes from each *C. burnetii* isolate for each class of antibiotics are compared to those same related proteins from other pathogens.

Bioinformatic Analysis of Putative Antibiotic Susceptibility Factors

Amino acid sequences of the genes identified in Table 3-1 were used to identify closely related proteins from a list of thirty-one other bacterial pathogens (Table 3-3). Sequences of these genes for each class of antibiotic were aligned with Clustal Omega and plotted on Dendroscope (EMBL-EBI Clustal Omega: ebi.ac.uk 2022; Huson and Scornavacca, 2012). Analysis of the *C. burnetii* strain genes in the context of related genes from thirty-one bacterial pathogens provides a higher resolution of where conservation of *C. burnetii* genes are highly clustered among the eight strains for genes involved in β-lactam susceptibility, macrolide susceptibility, quinolone susceptibility, streptogramin susceptibility, and tetracycline susceptibility.

Analysis of the putative β-lactam susceptibility proteins shows a tight clustering of the eight surveyed strains of *C. burnetii* (Figure 3-1). The closest branch is occupied by *L. pneumophila* with *P. aeruginosa* one branch further removed. Alignment of the β-lactamase family protein from the ten *C. burnetii* strains reveals a remarkably high degree of sequence conservation (Figure 3-2). Only two amino acid positions with

Table 3-3. List of bacterial pathogens from which proteins involved in antibiotic susceptibility were used in bioinformatic alignments with *C. burnetii* protein homologs.

Species	Gram +/-	Oxygen requirement	Intracellular/Extraceullar
Bacillus anthracis	+	Facultative anaerobic	Extracellular
Bartonella henselae	-	Aerobic	Facultative intracellular
Bordetella pertussis	-	Aerobic	Extracellular
Borrelia burgdorferi	-	Anaerobic	Extracellular
Brucella abortus	-	Aerobic	Intracellular
Chlamydia pneumoniae	-	Facultative/strictly aerobic	Obligate intracellular
Clostridium botulinum	+	Obligate anaerobic	Extracellular
Corynebacterium diphtheriae	+	Facultative anaerobic*	Extracellular
Coxiella burnetii	-	Facultative anaerobic	Obligate intracellular
Enterococcus faecalis	+	Facultative anaerobic	Extracellular
Escherichia coli	-	Facultative anaerobic	Intracellular/Extracellular
Francisella tularensis	-	Strictly aerobic	Facultative intracellular
Haemophilus influenzae	-		Extracellular
Helicobacter pylori	-	Microaerophile	Extracellular
Legionella pneumophila	-	Aerobic	Facultative intracellular
Leptospira interrogans	-	Strictly aerobic	Extracellular
Listeria monocytogenes	-	Facultative anaerobic	Intracellular
Mycobacterium tuberculosis		Aerobic	Extracellular
Mycoplasma pneumoniae		Aerobic	Extracellular
Neisseria gonorrhoeae	-	Aerobic	Facultative intracellular
Neisseria meningitidis	-	Aerobic	Extracellular
Pseudomonas aeruginosa	-	Obligate aerobic	Extracellular
Rickettsia rickettsii	-	Aerobic	Obligate intracellular
Salmonella enterica Typhi	-	Facultative anaerobic	Facultative intracellular
Shigella flexneri	-	Facultative anaerobic	Extracellular
Staphylococcus aureus	+	Facultative anaerobic	Facultative intracellular
Staphylococcus epidermidis	+	Facultative anaerobic	Extracellular
Streptococcus pneumoniae	+	Facultative anaerobic	Extracellular
Treponema pallidum	-	Aerobic	Extracellular
Ureaplasma urealyticum	-	Anaerobic	Extracellular
Vibrio cholerae	-	Facultative anaerobic	Extracellular
Yersinia pestis	-	Facultative anaerobic	Intracellular

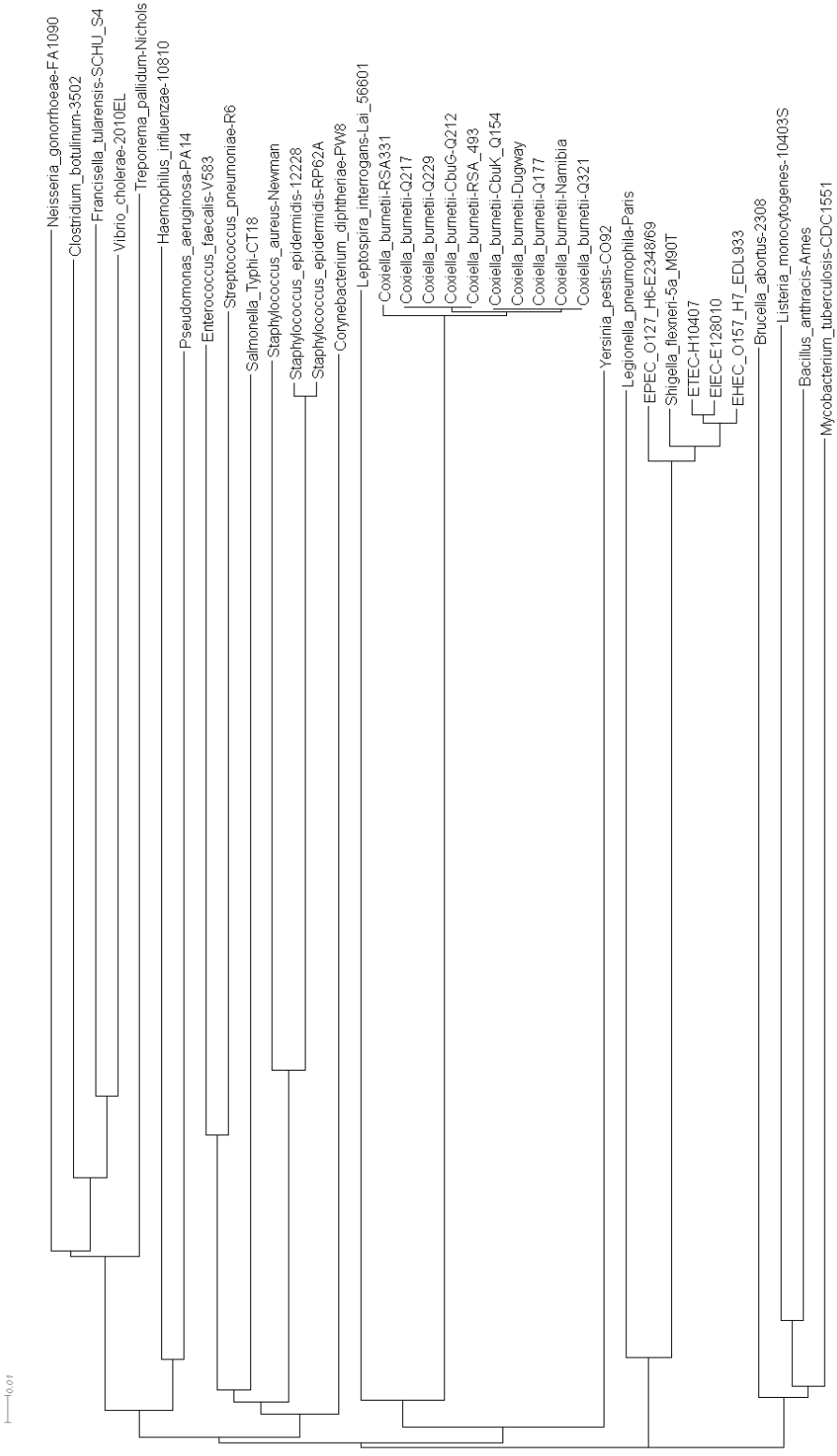

Figure 3-1. Dendroscopic Clustal Omega analysis of putative β-lactam susceptibility proteins. Compared with a panel of homologous proteins from panel of bacterial pathogens to reveal trends in relatedness of proteins.

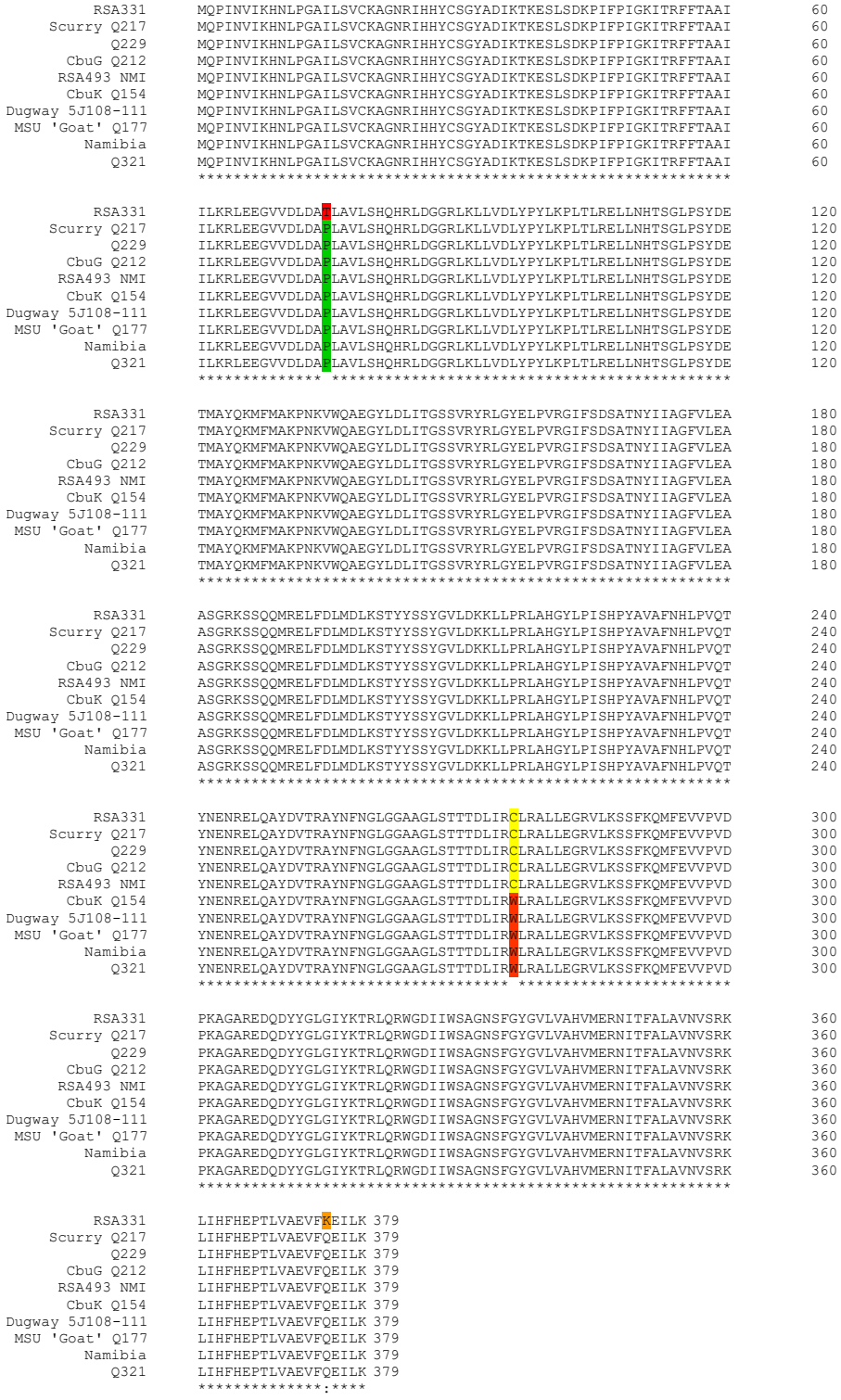

Figure 3-2. Alignment of the β-lactamase family protein from the ten *C. burnetii* strains.

* indicates identical residues, : indicates conserved residues.

unconserved amino acid variations (P75T and C276W) and one conserved amino acid variation (Q375K) within the β-lactamase protein sequences.

Analysis of the putative macrolide susceptibility proteins shows all ten surveyed strains of *C. burnetii* clustered on the same branch (Figure 3-3). The closest branch to this cluster. is occupied by *B. anthracis* with *P. aeruginosa* and *B. abortus* and *B. henselae*, one branch further removed. As was observed with the b-lactamase family protein sequence alignment, the amino acid alignment of the ten *C. burnetii* macrolide-efflux protein sequences revealed a high degree of conservation among all strains (Figure 3-4. One unconserved amino acid variation was observed, position P176L, in strain Q321 and one weakly conserved amino acid variation, position N257S, observed in RSA331 and NMI. Proteins from RSA331 and NMI also contain a conserved amino acid variation at position Q268K.

Once again, the next analysis shows all ten surveyed strains of *C. burnetii* putative quinolone susceptibility proteins clustered on the same branch (Figure 3-5). The closest branch includes *L. pneumophila* and *P. aeruginosa.* Analysis of the DNA gyrase subunit A amino acid sequences reveals a high level of conservation with only one weakly conserved amino acid variation, V262A and two conserved amino acid variations, H213Y (Figure 3-6). Studies identified amino acid substitutions in the DNA gyrase subunit A protein of *E. coli* that confer resistance to quinolones (Hopkins *et al.*, 2005; Muggeo *et al.*, 2020). One of those positions identified in *E. coli* is S80, which aligns with

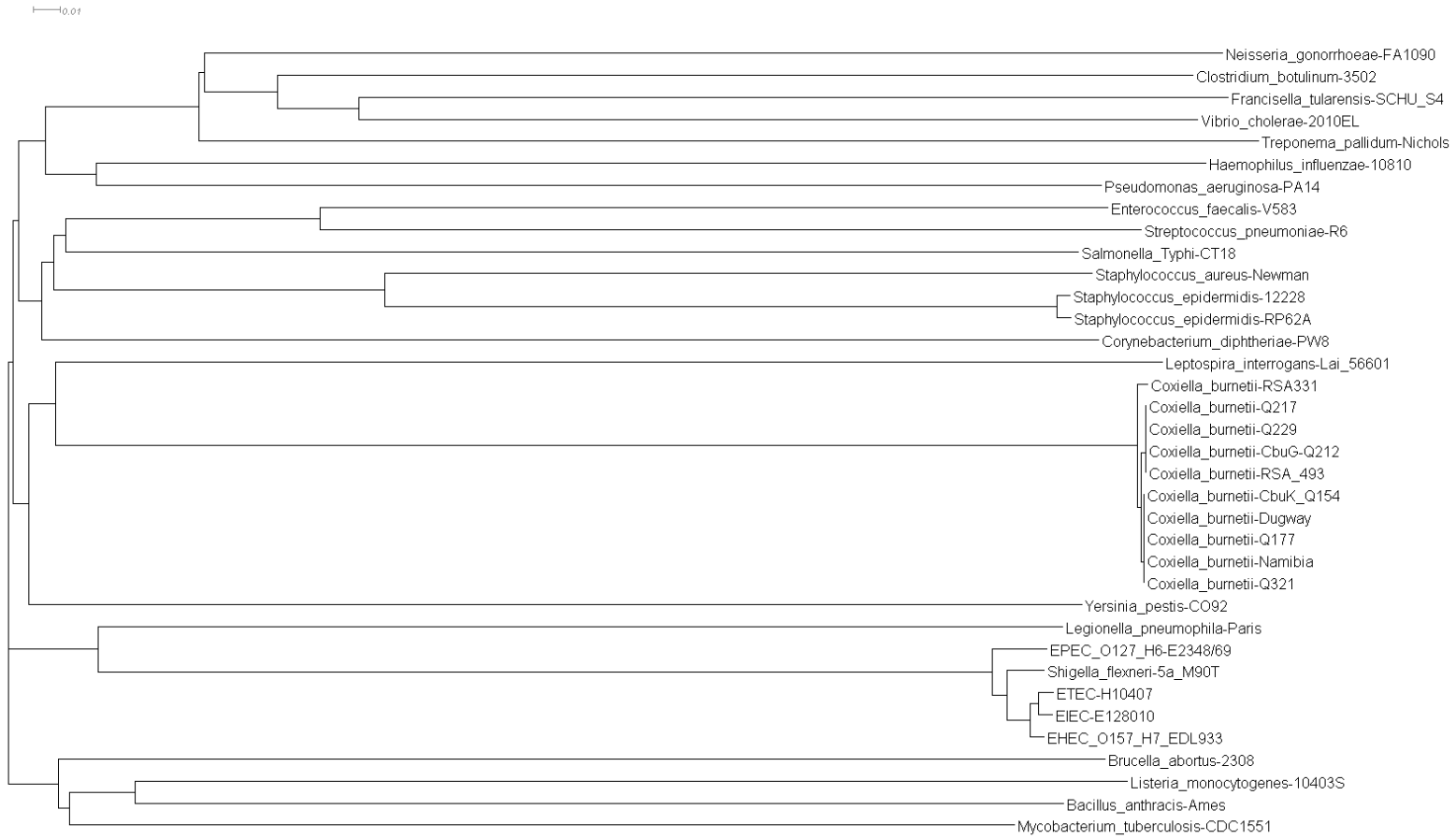

Figure 3-3. Dendroscopic Clustal Omega analysis of putative macrolide susceptibility proteins. Compared with a panel of homologous proteins from panel of bacterial pathogens to reveal trends in relatedness of proteins.

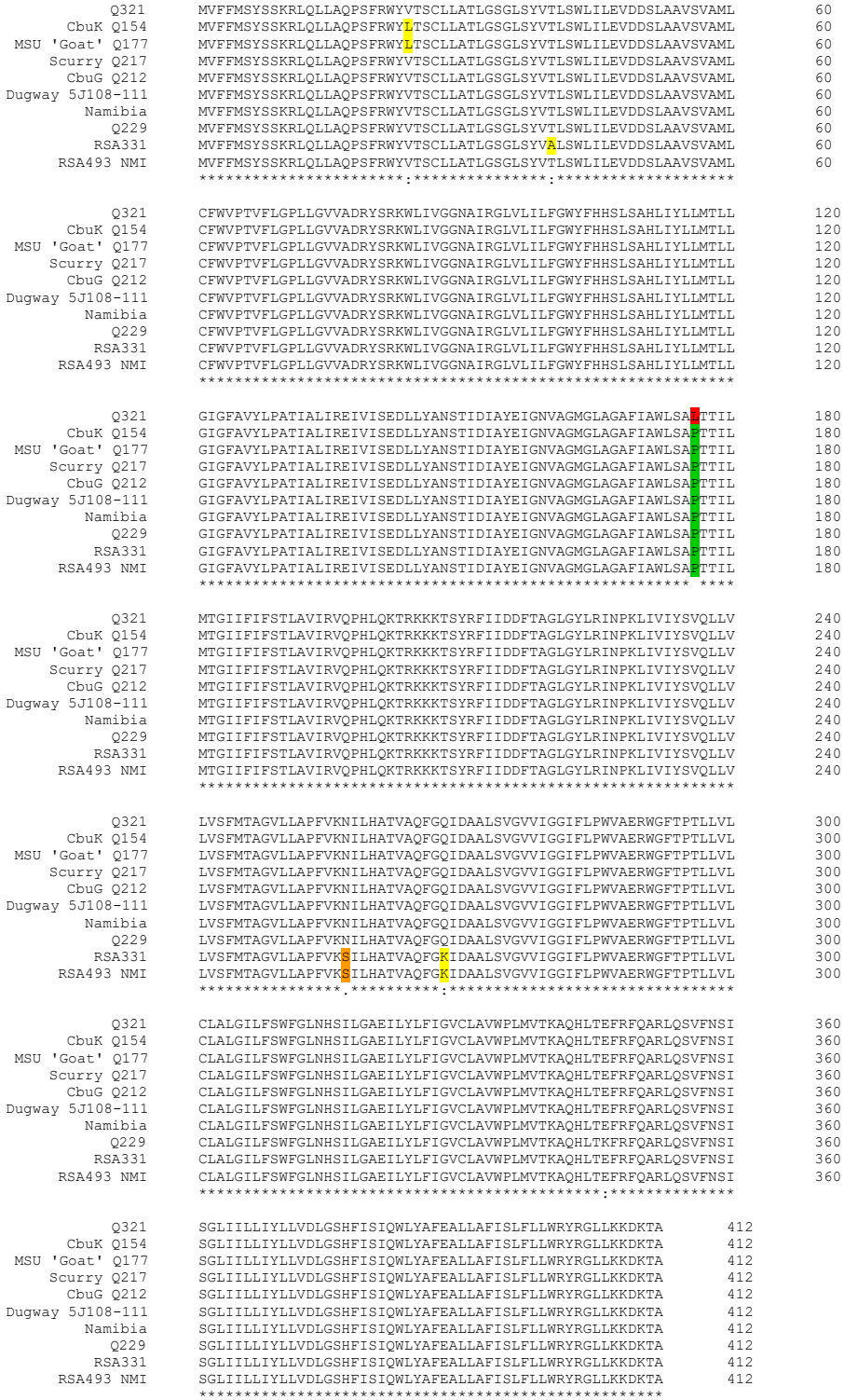

Figure 3-4. Alignment of the macrolide-efflux protein from the ten *C. burnetii* strains. * indicates identical residues, : and . indicate conserved and weakly conserved residues.

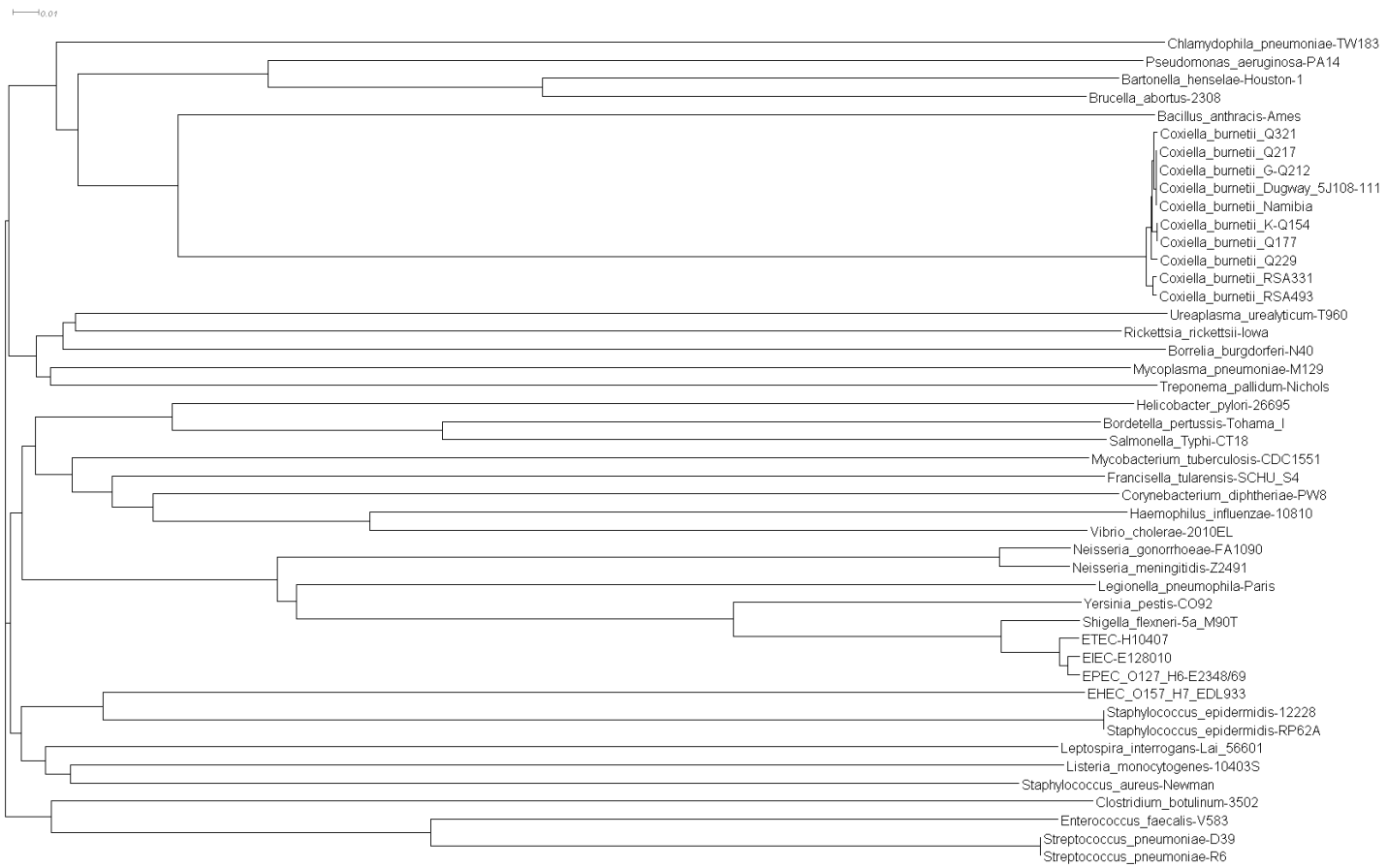

Figure 3-5. Dendroscopic analysis of Clustal Omega alignments of amino acid sequences of *C. burnetii* putative quinolone susceptibility proteins with panel of homologous proteins from panel of bacterial pathogens to reveal trends in relatedness of proteins.

```
          RSA331      MVMAEAAHEIIPITIEEELKQSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMSELGND        60
Dugway 5J108-111      MVMAEAAHEIIPITIEEELKQSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMSELGND        60
     Scurry Q217      MVMAEAAHEIIPITIEEELKQSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMSELGND        60
           Q229      MVMAEAAHEIIPITIEEELKQSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMSELGND        60
       CbuG Q212     MVMAEAAHEIIPITIEEELKQSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMSELGND        60
       CbuK Q154     MVMAEAAHEIIPITIEEELKQSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMSELGND        60
 MSU 'Goat' Q177     MVMAEAAHEIIPITIEEELKQSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMSELGND        60
         Namibia     MVMAEAAHEIIPITIEEELKQSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMSELGND        60
           Q321      MVMAEAAHEIIPITIEEELKQSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMSELGND        60
      RSA493 NMI     MVMAEAAHEIIPITIEEELKQSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMSELGND        60
                     ************************************************************

          RSA331      WNKPNKKSARIVGDVIGKYHPHGDVAVYDTIVRMAQPFSLRYLLIDGQGNFGSVDGDAPA        120
Dugway 5J108-111      WNKPNKKSARIVGDVIGKYHPHGDVAVYDTIVRMAQPFSLRYLLIDGQGNFGSVDGDAPA        120
     Scurry Q217      WNKPNKKSARIVGDVIGKYHPHGDVAVYDTIVRMAQPFSLRYLLIDGQGNFGSVDGDAPA        120
           Q229      WNKPNKKSARIVGDVIGKYHPHGDVAVYDTIVRMAQPFSLRYLLIDGQGNFGSVDGDAPA        120
       CbuG Q212     WNKPNKKSARIVGDVIGKYHPHGDVAVYDTIVRMAQPFSLRYLLIDGQGNFGSVDGDAPA        120
       CbuK Q154     WNKPNKKSARIVGDVIGKYHPHGDVAVYDTIVRMAQPFSLRYLLIDGQGNFGSVDGDAPA        120
 MSU 'Goat' Q177     WNKPNKKSARIVGDVIGKYHPHGDVAVYDTIVRMAQPFSLRYLLIDGQGNFGSVDGDAPA        120
         Namibia     WNKPNKKSARIVGDVIGKYHPHGDVAVYDTIVRMAQPFSLRYLLIDGQGNFGSVDGDAPA        120
           Q321      WNKPNKKSARIVGDVIGKYHPHGDVAVYDTIVRMAQPFSLRYLLIDGQGNFGSVDGDAPA        120
      RSA493 NMI     WNKPNKKSARIVGDVIGKYHPHGDVAVYDTIVRMAQPFSLRYLLIDGQGNFGSVDGDAPA        120
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          RSA331      AMRYTEIRLSRFAHALMADLDKETVDFAPNYDETEMAPAVLPTRVPNLLINGASGIAVGM        180
Dugway 5J108-111      AMRYTEIRLSRFAHALMADLDKETVDFAPNYDETEMAPAVLPTRVPNLLINGASGIAVGM        180
     Scurry Q217      AMRYTEIRLSRFAHALMADLDKETVDFAPNYDETEMAPAVLPTRVPNLLINGASGIAVGM        180
           Q229      AMRYTEIRLSRFAHALMADLDKETVDFAPNYDETEMAPAVLPTRVPNLLINGASGIAVGM        180
       CbuG Q212     AMRYTEIRLSRFAHALMADLDKETVDFAPNYDETEMAPAVLPTRVPNLLINGASGIAVGM        180
       CbuK Q154     AMRYTEIRLSRFAHALMADLDKETVDFAPNYDETEMAPAVLPTRVPNLLINGASGIAVGM        180
 MSU 'Goat' Q177     AMRYTEIRLSRFAHALMADLDKETVDFAPNYDETEMAPAVLPTRVPNLLINGASGIAVGM        180
         Namibia     AMRYTEIRLSRFAHALMADLDKETVDFAPNYDETEMAPAVLPTRVPNLLINGASGIAVGM        180
           Q321      AMRYTEIRLSRFAHALMADLDKETVDFAPNYDETEMAPAVLPTRVPNLLINGASGIAVGM        180
      RSA493 NMI     AMRYTEIRLSRFAHALMADLDKETVDFAPNYDETEMAPAVLPTRVPNLLINGASGIAVGM        180
                     ************************************************************

          RSA331      ATNIPPHNLNEIINATLALIENPDLNVEELMRHIPGPDFPTAGIINGRNGIVQAYKTGRG        240
Dugway 5J108-111      ATNIPPHNLNEIINATLALIENPDLNVEELMRHIPGPDFPTAGIINGRNGIVQAYKTGRG        240
     Scurry Q217      ATNIPPHNLNEIINATLALIENPDLNVEELMRYIPGPDFPTAGIINGRNGIVQAYKTGRG        240
           Q229      ATNIPPHNLNEIINATLALIENPDLNVEELMRYIPGPDFPTAGIINGRNGIVQAYKTGRG        240
       CbuG Q212     ATNIPPHNLNEIINATLALIENPDLNVEELMRYIPGPDFPTAGIINGRNGIVQAYKTGRG        240
       CbuK Q154     ATNIPPHNLNEIINATLALIENPDLNVEELMRHIPGPDFPTAGIINGRNGIVQAYKTGRG        240
 MSU 'Goat' Q177     ATNIPPHNLNEIINATLALIENPDLNVEELMRHIPGPDFPTAGIINGRNGIVQAYKTGRG        240
         Namibia     ATNIPPHNLNEIINATLALIENPDLNVEELMRHIPGPDFPTAGIINGRNGIVQAYKTGRG        240
           Q321      ATNIPPHNLNEIINATLALIENPDLNVEELMRHIPGPDFPTAGIINGRNGIVQAYKTGRG        240
      RSA493 NMI     ATNIPPHNLNEIINATLALIENPDLNVEELMRHIPGPDFPTAGIINGRNGIVQAYKTGRG        240
                     ********************************:***************************

          RSA331      RIYVRAKTEIETTKSGRSLIVVHELPYQVNKARLLEKIGELVREKRIEGISGLRDESDKR        300
Dugway 5J108-111      RIYVRAKTEIETTKSGRSLIVAHELPYQVNKARLLEKIGELVREKRIEGISGLRDESDKR        300
     Scurry Q217      RIYVRAKTEIETTKSGRSLIVVHELPYQVNKARLLEKIGELVREKRIEGISGLRDESDKR        300
           Q229      RIYVRAKTEIETTKSGRSLIVVHELPYQVNKARLLEKIGELVREKRIEGISGLRDESDKR        300
       CbuG Q212     RIYVRAKTEIETTKSGRSLIVVHELPYQVNKARLLEKIGELVREKRIEGISGLRDESDKR        300
       CbuK Q154     RIYVRAKTEIETTKSGRSLIVVHELPYQVNKARLLEKIGELVREKRIEGISGLRDESDKR        300
 MSU 'Goat' Q177     RIYVRAKTEIETTKSGRSLIVVHELPYQVNKARLLEKIGELVREKRIEGISGLRDESDKR        300
         Namibia     RIYVRAKTEIETTKSGRSLIVVHELPYQVNKARLLEKIGELVREKRIEGISGLRDESDKR        300
           Q321      RIYVRAKTEIETTKSGRSLIVVHELPYQVNKARLLEKIGELVREKRIEGISGLRDESDKR        300
      RSA493 NMI     RIYVRAKTEIETTKSGRSLIVVHELPYQVNKARLLEKIGELVREKRIEGISGLRDESDKR        300
                     ********************.***************************************

          RSA331      GMRMVIEVSRGDNAEIVLNNLYAQTQLQTVFGINMVALDNGQPRVLNLKQLLSAFLQHRR        360
Dugway 5J108-111      GMRMVIEVSRGDNAEIVLNNLYAQTQLQTVFGINMVALDNGQPRVLNLKQLLSAFLQHRR        360
     Scurry Q217      GMRMVIEVSRGDNAEIVLNNLYAQTQLQTVFGINMVALDNGQPRVLNLKQLLSAFLQHRR        360
           Q229      GMRMVIEVSRGDNAEIVLNNLYAQTQLQTVFGINMVALDNGQPRVLNLKQLLSAFLQHRR        360
       CbuG Q212     GMRMVIEVSRGDNAEIVLNNLYAQTQLQTVFGINMVALDNGQPRVLNLKQLLSAFLQHRR        360
       CbuK Q154     GMRMVIEVSRGDNAEIVLNNLYAQTQLQTVFGINMVALDNGQPRVLNLKQLLSAFLQHRR        360
 MSU 'Goat' Q177     GMRMVIEVSRGDNAEIVLNNLYAQTQLQTVFGINMVALDNGQPRVLNLKQLLSAFLQHRR        360
         Namibia     GMRMVIEVSRGDNAEIVLNNLYAQTQLQTVFGINMVALDNGQPRVLNLKQLLSAFLQHRR        360
           Q321      GMRMVIEVSRGDNAEIVLNNLYAQTQLQTVFGINMVALDNGQPRVLNLKQLLSAFLQHRR        360
      RSA493 NMI     GMRMVIEVSRGDNAEIVLNNLYAQTQLQTVFGINMVALDNGQPRVLNLKQLLSAFLQHRR        360
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          RSA331      EVVTRRTLFELRKARERAHILEGLGVALANIDEVIALIKKAKTPAIAKENLLAQAWKPGA        420
Dugway 5J108-111      EVVTRRTLFELRKARERAHILEGLGVALANIDEVIALIKKAKTPAIAKENLLAQAWKPGA        420
     Scurry Q217      EVVTRRTLFELRKARERAHILEGLGVALANIDEVIALIKKAKTPAIAKENLLAQAWKPGA        420
           Q229      EVVTRRTLFELRKARERAHILEGLGVALANIDEVIALIKKAKTPAIAKENLLAQAWKPGA        420
       CbuG Q212     EVVTRRTLFELRKARERAHILEGLGVALANIDEVIALIKKAKTPAIAKENLLAQAWKPGA        420
       CbuK Q154     EVVTRRTLFELRKARERAHILEGLGVALANIDEVIALIKKAKTPAIAKENLLAQAWKPGA        420
 MSU 'Goat' Q177     EVVTRRTLFELRKARERAHILEGLGVALANIDEVIALIKKAKTPAIAKENLLAQAWKPGA        420
         Namibia     EVVTRRTLFELRKARERAHILEGLGVALANIDEVIALIKKAKTPAIAKENLLAQAWKPGA        420
           Q321      EVVTRRTLFELRKARERAHILEGLGVALANIDEVIALIKKAKTPAIAKENLLAQAWKPGA        420
      RSA493 NMI     EVVTRRTLFELRKARERAHILEGLGVALANIDEVIALIKKAKTPAIAKENLLAQAWKPGA        420
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          RSA331      VADFLKKAGSDRTQPDDLAAEFGLRKEGYYLSPAQAQAILDLRLHRLTGLETDKIREEYI        480
Dugway 5J108-111      VADFLKKAGSDRTRPDDLAAEFGLRKEGYYLSPAQAQAILDLRLHRLTGLETDKIREEYI        480
     Scurry Q217      VADFLKKAGSDRTRPDDLAAEFGLRKEGYYLSPAQAQAILDLRLHRLTGLETDKIREEYI        480
           Q229      VADFLKKAGSDRTRPDDLAAEFGLRKEGYYLSPAQAQAILDLRLHRLTGLETDKIREEYI        480
       CbuG Q212     VADFLKKAGSDRTRPDDLAAEFGLRKEGYYLSPAQAQAILDLRLHRLTGLETDKIREEYI        480
       CbuK Q154     VADFLKKAGSDRTRPDDLAAEFGLRKEGYYLSPAQAQAILDLRLHRLTGLETDKIREEYI        480
 MSU 'Goat' Q177     VADFLKKAGSDRTRPDDLAAEFGLRKEGYYLSPAQAQAILDLRLHRLTGLETDKIREEYI        480
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         Namibia      VADFLKKAGSDRTRPDDLAAEFGLRKEGYYLSPAQAQAILDLRLHRLTGLETDKIREEYI        480
           Q321       VADFLKKAGSDRTRPDDLAAEFGLRKEGYYLSPAQAQAILDLRLHRLTGLETDKIREEYI        480
       RSA493 NMI     VADFLKKAGSDRTRPDDLAAEFGLRKEGYYLSPAQAQAILDLRLHRLTGLETDKIREEYI        480
                      ************:*********************************************

          RSA331      AIIDKIEELIAIVSDPDKLHEVIREELIAVKEQFGDERRTVIIDDHSDLTHEDLIPEEHR        540
Dugway 5J108-111      AIIDKIEELIAIVSDPDKLHEVIREELIAVKEQFGDERRTVIIDDHSDLTHEDLIPEEHR        540
     Scurry Q217      AIIDKIEELIAIVSDPDKLHEVIREELIAVKEQFGDERRTVIIDDHSDLTHEDLIPEEHR        540
           Q229       AIIDKIEELIAIVSDPDKLHEVIREELIAVKEQFGDERRTVIIDDHSDLTHEDLIPEEHR        540
        CbuG Q212     AIIDKIEELIAIVSDPDKLHEVIREELIAVKEQFGDERRTVIIDDHSDLTHEDLIPEEHR        540
        CbuK Q154     AIIDKIEELIAIVSDPDKLHEVIREELIAVKEQFGDERRTVIIDDHSDLTHEDLIPEEHR        540
 MSU 'Goat' Q177      AIIDKIEELIAIVSDPDKLHEVIREELIAVKEQFGDERRTVIIDDHSDLTHEDLIPEEHR        540
         Namibia      AIIDKIEELIAIVSDPDKLHEVIREELIAVKEQFGDERRTVIIDDHSDLTHEDLIPEEHR        540
           Q321       AIIDKIEELIAIVSDPDKLHEVIREELIAVKEQFGDERRTVIIDDHSDLTHEDLIPEEHR        540
       RSA493 NMI     AIIDKIEELIAIVSDPDKLHEVIREELIAVKEQFGDERRTVIIDDHSDLTHEDLIPEEHR        540
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          RSA331      VVTLSHEGYIKSQSLSSYQAQHRGGRGKLAAAVKEQDFVKNILVANSHDTILCFSTQGKV        600
Dugway 5J108-111      VVTLSHEGYIKSQSLSSYQAQHRGGRGKLAAAVKEQDFVKNILVANSHDTILCFSTQGKV        600
     Scurry Q217      VVTLSHEGYIKSQSLSSYQAQHRGGRGKLAAAVKEQDFVKNILVANSHDTILCFSTQGKV        600
           Q229       VVTLSHEGYIKSQSLSSYQAQHRGGRGKLAAAVKEQDFVKNILVANSHDTILCFSTQGKV        600
        CbuG Q212     VVTLSHEGYIKSQSLSSYQAQHRGGRGKLAAAVKEQDFVKNILVANSHDTILCFSTQGKV        600
        CbuK Q154     VVTLSHEGYIKSQSLSSYQAQHRGGRGKLAAAVKEQDFVKNILVANSHDTILCFSTQGKV        600
 MSU 'Goat' Q177      VVTLSHEGYIKSQSLSSYQAQHRGGRGKLAAAVKEQDFVKNILVANSHDTILCFSTQGKV        600
         Namibia      VVTLSHEGYIKSQSLSSYQAQHRGGRGKLAAAVKEQDFVKNILVANSHDTILCFSTQGKV        600
           Q321       VVTLSHEGYIKSQSLSSYQAQHRGGRGKLAAAVKEQDFVKNILVANSHDTILCFSTQGKV        600
       RSA493 NMI     VVTLSHEGYIKSQSLSSYQAQHRGGRGKLAAAVKEQDFVKNILVANSHDTILCFSTQGKV        600
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          RSA331      YWLKVYQVPQGSRIARGRPIINLLPLVKDEQISAILPIRAYDGSHFVFMATAQGAIKKVS        660
Dugway 5J108-111      YWLKVYQVPQGSRIARGRPIINLLPLVKDEQISAILPIRAYDGSHFVFMATAQGAIKKVS        660
     Scurry Q217      YWLKVYQVPQGSRIARGRPIINLLPLVKDEQISAILPIRAYDGSHFVFMATAQGAIKKVS        660
           Q229       YWLKVYQVPQGSRIARGRPIINLLPLVKDEQISAILPIRAYDGSHFVFMATAQGAIKKVS        660
        CbuG Q212     YWLKVYQVPQGSRIARGRPIINLLPLVKDEQISAILPIRAYDGSHFVFMATAQGAIKKVS        660
        CbuK Q154     YWLKVYQVPQGSRIARGRPIINLLPLVKDEQISAILPIRAYDGSHFVFMATAQGAIKKVS        660
 MSU 'Goat' Q177      YWLKVYQVPQGSRIARGRPIINLLPLVKDEQISAILPIRAYDGSHFVFMATAQGAIKKVS        660
         Namibia      YWLKVYQVPQGSRIARGRPIINLLPLVKDEQISAILPIRAYDGSHFVFMATAQGAIKKVS        660
           Q321       YWLKVYQVPQGSRIARGRPIINLLPLVKDEQISAILPIRAYDGSHFVFMATAQGAIKKVS        660
       RSA493 NMI     YWLKVYQVPQGSRIARGRPIINLLPLVKDEQISAILPIRAYDGSHFVFMATAQGAIKKVS        660
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          RSA331      LAEFSQPRTKGKIALALNEGDRLVGVDITDGKKEIMLVTDAGKAIRFHEKEVREMGRSAR        720
Dugway 5J108-111      LAEFSQPRTKGKIALALNEGDRLVGVDITDGKKEIMLVTDAGKAIRFHEKEVREMGRSAR        720
     Scurry Q217      LAEFSQPRTKGKIALALNEGDRLVGVDITDGKKEIMLVTDAGKAIRFHEKEVREMGRSAR        720
           Q229       LAEFSQPRTKGKIALALNEGDRLVGVDITDGKKEIMLVTDAGKAIRFHEKEVREMGRSAR        720
        CbuG Q212     LAEFSQPRTKGKIALALNEGDRLVGVDITDGKKEIMLVTDAGKAIRFHEKEVREMGRSAR        720
        CbuK Q154     LAEFSQPRTKGKIALALNEGDRLVGVDITDGKKEIMLVTDAGKAIRFHEKEVREMGRSAR        720
 MSU 'Goat' Q177      LAEFSQPRTKGKIALALNEGDRLVGVDITDGKKEIMLVTDAGKAIRFHEKEVREMGRSAR        720
         Namibia      LAEFSQPRTKGKIALALNEGDRLVGVDITDGKKEIMLVTDAGKAIRFHEKEVREMGRSAR        720
           Q321       LAEFSQPRTKGKIALALNEGDRLVGVDITDGKKEIMLVTDAGKAIRFHEKEVREMGRSAR        720
       RSA493 NMI     LAEFSQPRTKGKIALALNEGDRLVGVDITDGKKEIMLVTDAGKAIRFHEKEVREMGRSAR        720
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          RSA331      GVRGIKLKAKQNVIALIVVKPKGNILTATVHGYGQRTALDDYRSTGRGGQGVMAIRINSR        780
Dugway 5J108-111      GVRGIKLKAKQNVIALIVVKPKGNILTATVHGYGQRTALDDYRSTGRGGQGVMAIRINSR        780
     Scurry Q217      GVRGIKLKAKQNVIALIVVKPKGNILTATVHGYGQRTALDDYRSTGRGGQGVMAIRINSR        780
           Q229       GVRGIKLKAKQNVIALIVVKPKGNILTATVHGYGQRTALDDYRSTGRGGQGVMAIRINSR        780
        CbuG Q212     GVRGIKLKAKQNVIALIVVKPKGNILTATVHGYGQRTALDDYRSTGRGGQGVMAIRINSR        780
        CbuK Q154     GVRGIKLKAKQNVIALIVVKPKGNILTATVHGYGQRTALDDYRSTGRGGQGVMAIRINSR        780
  MSU 'Goat' Q177     GVRGIKLKAKQNVIALIVVKPKGNILTATVHGYGQRTALDDYRSTGRGGQGVMAIRINSR        780
         Namibia      GVRGIKLKAKQNVIALIVVKPKGNILTATVHGYGQRTALDDYRSTGRGGQGVMAIRINSR        780
           Q321       GVRGIKLKAKQNVIALIVVKPKGNILTATVHGYGQRTALDDYRSTGRGGQGVMAIRINSR        780
       RSA493 NMI     GVRGIKLKAKQNVIALIVVKPKGNILTATVHGYGQRTALDDYRSTGRGGQGVMAIRINSR        780
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          RSA331      NGKVVSAAQVFDDDDVLLISDKGTLVRTRVNEISQMGRNTQGVRLIQLSQDELLVGMEAI        840
Dugway 5J108-111      NGKVVSAAQVFDDDDVLLISDKGTLVRTRVNEISQMGRNTQGVRLIQLSQDELLVGMEAI        840
     Scurry Q217      NGKVVSAAQVFDDDDVLLISDKGTLVRTRVNEISQMGRNTQGVRLIQLSQDELLVGMEAI        840
           Q229       NGKVVSAAQVFDDDDVLLISDKGTLVRTRVNEISQMGRNTQGVRLIQLSQDELLVGMEAI        840
        CbuG Q212     NGKVVSAAQVFDDDDVLLISDKGTLVRTRVNEISQMGRNTQGVRLIQLSQDELLVGMEAI        840
        CbuK Q154     NGKVVSAAQVFDDDDVLLISDKGTLVRTRVNEISQMGRNTQGVRLIQLSQDELLVGMEAI        840
 MSU 'Goat' Q177      NGKVVSAAQVFDDDDVLLISDKGTLVRTRVNEISQMGRNTQGVRLIQLSQDELLVGMEAI        840
         Namibia      NGKVVSAAQVFDDDDVLLISDKGTLVRTRVNEISQMGRNTQGVRLIQLSQDELLVGMEAI        840
           Q321       NGKVVSAAQVFDDDDVLLISDKGTLVRTRVNEISQMGRNTQGVRLIQLSQDELLVGMEAI        840
       RSA493 NMI     NGKVVSAAQVFDDDDVLLISDKGTLVRTRVNEISQMGRNTQGVRLIQLSQDELLVGMEAI        840
                      ************************************************************

          RSA331      SAELIEEPSP          850
Dugway 5J108-111      SAELIEEPSP          850
     Scurry Q217      SAELIEEPSP          850
           Q229       SAELIEEPSP          850
        CbuG Q212     SAELIEEPSP          850
        CbuK Q154     SAELIEEPSP          850
 MSU 'Goat' Q177      SAELIEEPSP          850
         Namibia      SAELIEEPSP          850
           Q321       SAELIEEPSP          850
       RSA493 NMI     SAELIEEPSP          850
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Figure 3-6. Alignment of the DNA gyrase subunitA protein from the ten *C. burnetii* strains. * indicates identical residues, : and . indicate conserved and weakly conserved residues.

V85 (highlighted in green in Fig. 3-6), a potential quinolone resistance allele for *C. burnetii.*

Analysis of the putative streptogramin susceptibility proteins shows the protein sequences from the ten surveyed strains of *C. burnetii* clustered on a single branch (Figure 3-7). The closest branch includes proteins from *S. epidermidis* strains 12228 and RP62A. Analysis of the protein sequence alignments from the ten *C. burnetii* streptogramin A acetyltransferase-like proteins reveals only one unconserved amino acid variation in Q321 at position D48Y (Figure 3-8). One conserved amino acid variation at Q14K was only observed in proteins from strains CbuK Q154 and MSU 'Goat' Q177. Another conserved amino acid variation at H95Y was only observed in proteins from strains RSA331 and NMI. Two additional conserved amino acid variations were observed at positions I96M and V112L in proteins from strains Dugway 5J108-111 and Namibia, respectively.

Analysis of the putative tetracycline susceptibility TypA GTP-binding proteins shows proteins from all tell surveyed strains of *C. burnetii* clustered on a single branch (Figure 3-9). The closest branch is only occupied by *L. pneumophila*. More weakly conserved amino acid variations are observed in the alignment of the TypA GTP-binding protein sequences from the ten *C. burnetii* strains compared to other amino acids analyzed (Figure 3-10). Weakly conserved amino acid variations are found in proteins from RSA331 and NMI at position A195V. Protein from the Namibia strain has a weakly conserved amino acid variation at position T230K. Additionally, proteins from the ten

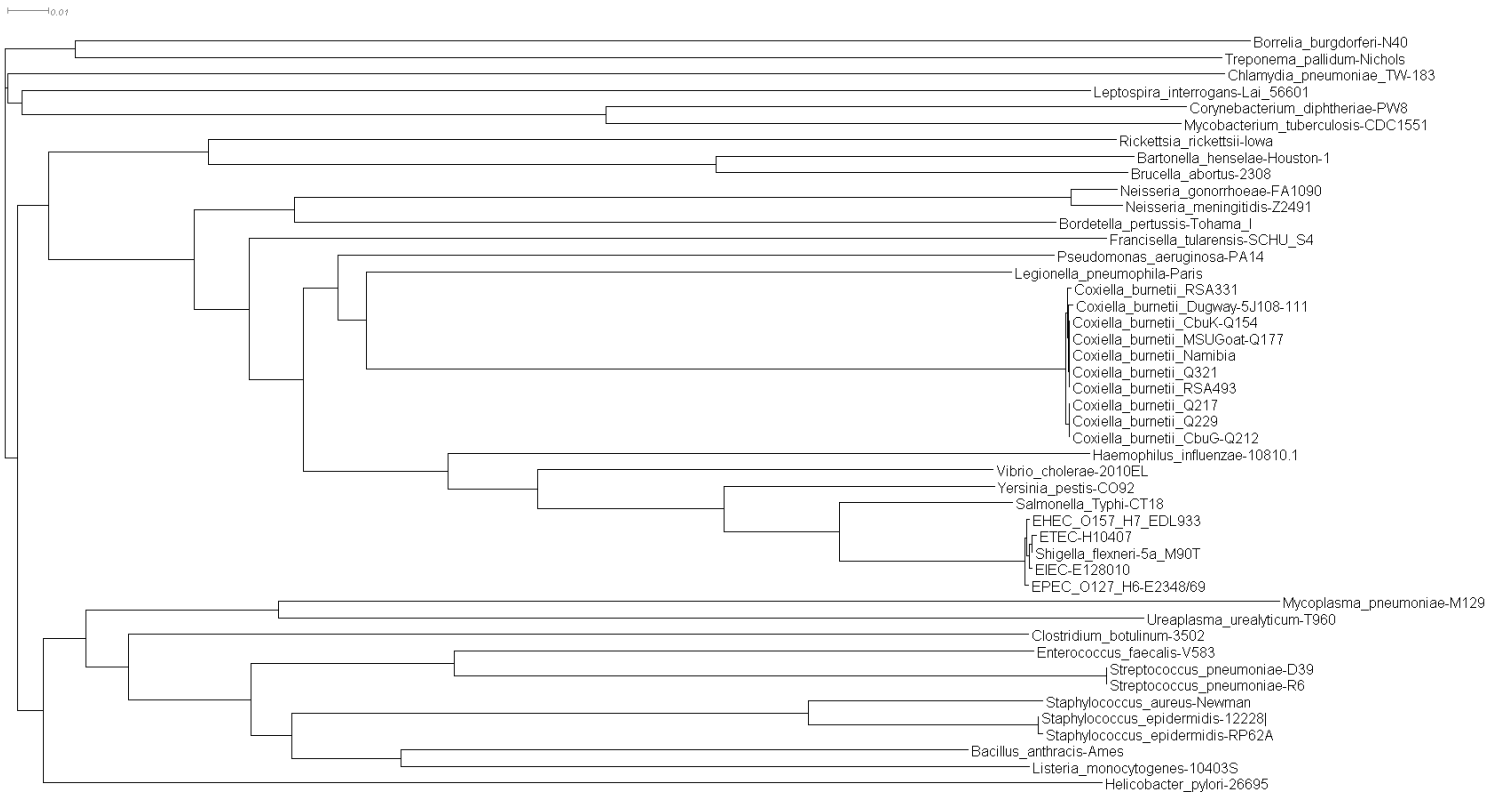

Figure 3-7. Dendroscopic analysis of Clustal Omega alignments of amino acid sequences of *C. burnetii* putative streptogramin susceptibility proteins with panel of homologous proteins from panel of bacterial pathogens to reveal trends in relatedness of proteins.

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          RSA331      MSHIFDELDNAYKQKEAYLLDHWNRSLPFQDAMFDRWERAQRLGFGEDVSIYNSALVFGN        60
       RSA493 NMI     MSHIFDELDNAYKQKEAYLLDHWNRSLPFQDAMFDRWERAQRLGFGEDVSIYNSALVFGN        60
             Q321      MSHIFDELDNAYKQKEAYLLDHWNRSLPFQDAMFDRWERAQRLGFGERVSIYNSALVFGN        60
          Namibia      MSHIFDELDNAYKQKEAYLLDHWNRSLPFQDAMFDRWERAQRLGFGEDVSIYNSALVFGN        60
  Dugway 5J108-111     MSHIFDELDNAYKQKEAYLLDHWNRSLPFQDAMFDRWERAQRLGFGEDVSIYNSALVFGN        60
       Scurry Q217     MSHIFDELDNAYKQKEAYLLDHWNRSLPFQDAMFDRWERAQRLGFGEDVSIYNSALVFGN        60
             Q229      MSHIFDELDNAYKQKEAYLLDHWNRSLPFQDAMFDRWERAQRLGFGEDVSIYNSALVFGN        60
         CbuG Q212     MSHIFDELDNAYKQKEAYLLDHWNRSLPFQDAMFDRWERAQRLGFGEDVSIYNSALVFGN        60
         CbuK Q154     MSHIFDELDNAYKKKEAYLLDHWNRSLPFQDAMFDRWERAQRLGFGEDVSIYNSALVFGN        60
   MSU 'Goat' Q177     MSHIFDELDNAYKKKEAYLLDHWNRSLPFQDAMFDRWERAQRLGFGEDVSIYNSALVFGN        60
                       ************:********************************* ************

          RSA331      VAVGANSWIGPYVILDGSGGRLSIGCYCSISAGVYIYTHDSVAWAVTGGKSVYQKGDVTI        120
       RSA493 NMI     VAVGANSWIGPYVILDGSGGRLSIGCYCSISAGVYIYTHDSVAWAVTGGKSVYQKGDVTI        120
             Q321      VAVGANSWIGPYVILDGSGGRLSIGCYCSISAGVHIYTHDSVAWAVTGGKSVYQKGDVTI        120
          Namibia      VAVGANSWIGPYVILDGSGGRLSIGCYCSISAGVHIYTHDSVAWAVTGGKSLYQKGDVTI        120
  Dugway 5J108-111     VAVGANSWIGPYVILDGSGGRLSIGCYCSISAGVHMYTHDSVAWAVTGGKSVYQKGDVTI        120
       Scurry Q217     VAVGANSWIGPYVILDGSGGRLSIGCYCSISAGVHIYTHDSVAWAVTGGKSVYQKGDVTI        120
             Q229      VAVGANSWIGPYVILDGSGGRLSIGCYCSISAGVHIYTHDSVAWAVTGGKSVYQKGDVTI        120
         CbuG Q212     VAVGANSWIGPYVILDGSGGRLSIGCYCSISAGVHIYTHDSVAWAVTGGKSVYQKGDVTI        120
         CbuK Q154     VAVGANSWIGPYVILDGSGGRLSIGCYCSISAGVHIYTHDSVAWAVTGGKSVYQKGDVTI        120
   MSU 'Goat' Q177     VAVGANSWIGPYVILDGSGGRLSIGCYCSISAGVHIYTHDSVAWAVTGGKSVYQKGDVTI        120
                       **********************************::***************:********

          RSA331      GNCCYIAPQSIIKMGIKIGDHSIIGANSFVNTNVPAYSIVAGSPAKVIGKVEIINDKVNL        180
       RSA493 NMI     GNCCYIAPQSIIKMGIKIGDHSIIGANSFVNTNVPAYSIVAGSPAKVIGKVEIINDKVNL        180
             Q321      GNCCYIAPQSIIKMGIKIGDHSIIGANSFVNTNVPAYSIVAGSPAKVIGKVEIINDKVNL        180
          Namibia      GNCCYIAPQSIIKMGIKIGDHSIIGANSFVNTNVPAYSIVAGSPAKVIGKVEIINDKVNL        180
  Dugway 5J108-111     GNCCYIAPQSIIKMGIKIGDHSIIGANSFVNTNVPAYSIVAGSPAKVIGKVEIINDKVNL        180
       Scurry Q217     GNCCYIAPQSIIKMGIKIGDHSIIGANSFVNTNVPAYSIVAGSPAKVIGKVEIINDKVNL        180
             Q229      GNCCYIAPQSIIKMGIKIGDHSIIGANSFVNTNVPAYSIVAGSPAKVIGKVEIINDKVNL        180
         CbuG Q212     GNCCYIAPQSIIKMGIKIGDHSIIGANSFVNTNVPAYSIVAGSPAKVIGKVEIINDKVNL        180
         CbuK Q154     GNCCYIAPQSIIKMGIKIGDHSIIGANSFVNTNVPAYSIVAGSPAKVIGKVEIINDKVNL        180
   MSU 'Goat' Q177     GNCCYIAPQSIIKMGIKIGDHSIIGANSFVNTNVPAYSIVAGSPAKVIGKVEIINDKVNL        180
                       ************************************************************

          RSA331      KYY       183
       RSA493 NMI     KYY       183
             Q321      KYY       183
          Namibia      KYY       183
  Dugway 5J108-111     KYY       183
       Scurry Q217     KYY       183
             Q229      KYY       183
         CbuG Q212     KYY       183
         CbuK Q154     KYY       183
   MSU 'Goat' Q177     KYY       183
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Figure 3-8. Alignment of the streptogramin A acetyltransferase-like protein from the ten *C. burnetii* strains. * indicates identical residues, : and . indicate conserved and weakly conserved residues.

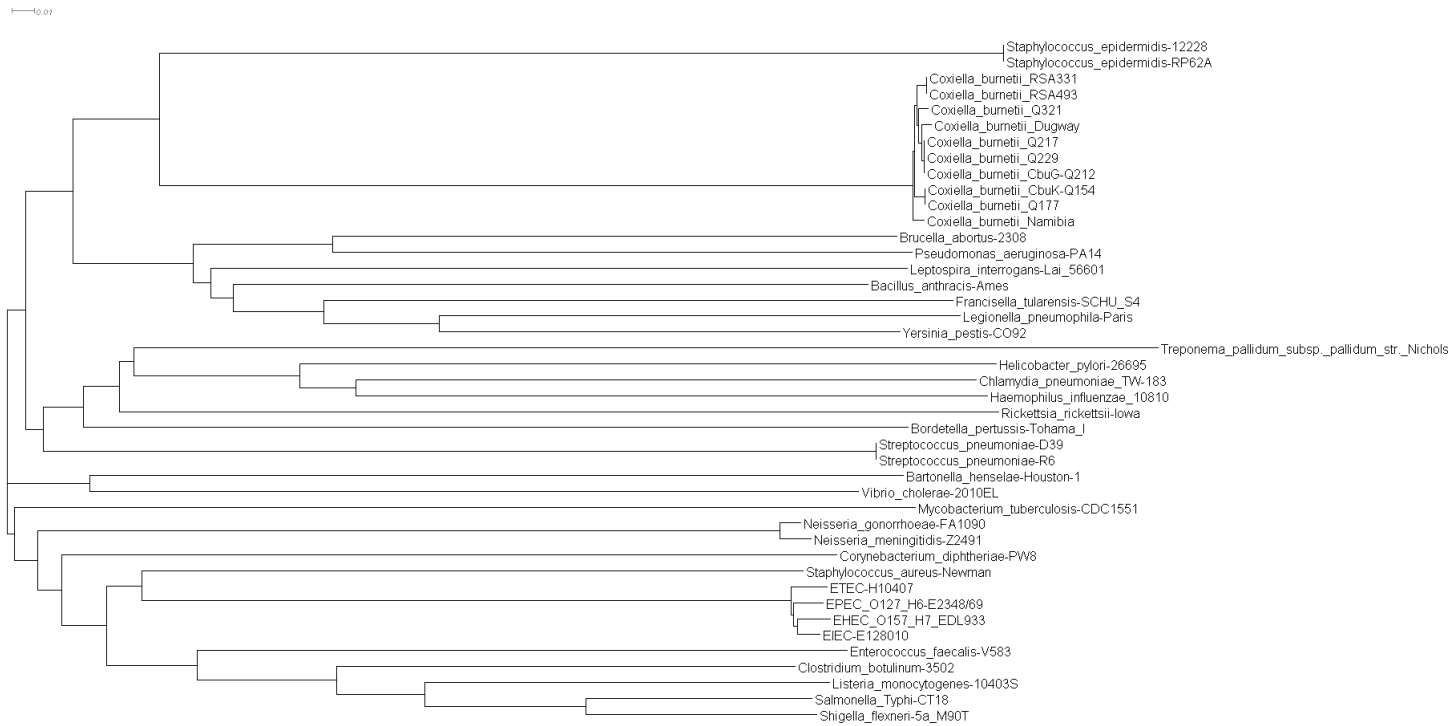

Figure 3-9. Dendroscopic analysis of Clustal Omega alignments of amino acid sequences of *C. burnetii* putative tetracycline susceptibility proteins with panel of homologous proteins from panel of bacterial pathogens to reveal trends in relatedness of proteins.

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          RSA331      MIENIRNIAIIAHVDHGKTTLVDQLLQQSGTLNERAAPVERMMDSNILERERGITILAKN        60
       RSA493 NMI     MIENIRNIAIIAHVDHGKTTLVDQLLQQSGTLNERAAPVERMMDSNILERERGITILAKN        60
      Scurry Q217     MIENIRNIAIIAHVDHGKTTLVDQLLQQSGTLNERAAPVERMMDSNILERERGITILAKN        60
            Q229      MIENIRNIAIIAHVDHGKTTLVDQLLQQSGTLNERAAPVERMMDSNILERERGITILAKN        60
         CbuG Q212    MIENIRNIAIIAHVDHGKTTLVDQLLQQSGTLNERAAPVERMMDSNILERERGITILAKN        60
  Dugway 5J108-111    MIENIRNIAIIAHVDHGKTTLVDQLLQQSGTLNERAAPVERMMDSNILERERGITILAKN        60
          Namibia     MIENIRNIAIIAHVDHGKTTLVDQLLQQSGTLNERAAPVERMMDSNILERERGITILAKN        60
         CbuK Q154    MIENIRNIAIIAHVDHGKTTLVDQLLQQSGTLNERAAPVERMMDSNILERERGITILAKN        60
   MSU 'Goat' Q177    MIENIRNIAIIAHVDHGKTTLVDQLLQQSGTLNERAAPVERMMDSNILERERGITILAKN        60
            Q321      MIENIRNIAIIAHVDHGKTTLVDQLLQQSGTLNERAAPVERMMDSNILERERGITILAKN        60
                      ************************************************************

          RSA331      TAIRWRNYRININVDTPGHADFGGEVERILSMVDSVLLLVDAVEGPMPQTRFVTRKAFSWG        120
       RSA493 NMI     TAIRWRNYRININVDTPGHADFGGEVERILSMVDSVLLLVDAVEGPMPQTRFVTRKAFSWG        120
      Scurry Q217     TAIRWRNYRININVDTPGHADFGGEVERILSMVDSVLLLVDAVEGPMPQTRFVTRKAFSWG        120
            Q229      TAIRWRNYRININVDTPGHADFGGEVERILSMVDSVLLLVDAVEGPMPQTRFVTRKAFSWG        120
         CbuG Q212    TAIRWRNYRININVDTPGHADFGGEVERILSMVDSVLLLVDAVEGPMPQTRFVTRKAFSWG        120
  Dugway 5J108-111    TAIRWRNYRININVDTPGHADFGGEVERILSMVDSVLLLVDAVEGPMPQTRFVTRKAFSWG        120
          Namibia     TAIRWRNYRININVDTPGHADFGGEVERILSMVDSVLLLVDAVEGPMPQTRFVTRKAFSWG        120
         CbuK Q154    TAIRWRNYRININVDTPGHADFGGEVERILSMVDSVLLLVDAVEGPMPQTRFVTRKAFSWG        120
   MSU 'Goat' Q177    TAIRWRNYRININVDTPGHADFGGEVERILSMVDSVLLLVDAVEGPMPQTRFVTRKAFSWG        120
            Q321      TAIRWRNYRININVDTPGHADFGGEVERILSMVDSVLLLVDAVEGPMPQTRFVTRKAFSWG        120
                      ************************************************************

          RSA331      LKPIVVVNKIDRPGARPDWVVEQVFDLFVSLDATDAQLDFPVVYASALKGYATLDLSHPS        180
       RSA493 NMI     LKPIVVVNKIDRPGARPDWVVEQVFDLFVSLDATDAQLDFPVVYASALKGYATLDLSHPS        180
      Scurry Q217     LKPIVVVNKIDRPGARPDWVVEQVFDLFVSLDATDAQLDFPVVYASALKGYATLDLSHPS        180
            Q229      LKPIVVVNKIDRPGARPDWVVEQVFDLFVSLDATDAQLDFPVVYASALKGYATLDLSHPS        180
         CbuG Q212    LKPIVVVNKIDRPGARPDWVVEQVFDLFVSLDATDAQLDFPVVYASALKGYATLDLSHPS        180
  Dugway 5J108-111    LKPIVVVNKIDRPGARPDWVVEQVFDLFVSLDATDAQLDFPVVYASALKGYATLDLSHPS        180
          Namibia     LKPIVVVNKIDRPGARPDWVVEQVFDLFVSLDATDAQLDFPVVYASALKGYATLDLSHPS        180
         CbuK Q154    LKPIVVVNKIDRPGARPDWVVEQVFDLFVSLDATDAQLDFPVVYASALKGYATLDLSHPS        180
   MSU 'Goat' Q177    LKPIVVVNKIDRPGARPDWVVEQVFDLFVSLDATDAQLDFPVVYASALKGYATLDLSHPS        180
            Q321      LKPIVVVNKIDRPGARPDWVVEQVFDLFVSLDATDAQLDFPVVYASALKGYATLDLSHPS        180
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          RSA331      TDMTPLFETIVSKVVPPQVDLNGPFQMQISSLDYSSYVGAIGIGRIQRGTIRRNTPVIII        240
       RSA493 NMI     TDMTPLFETIVSKVVPPQVDLNGPFQMQISSLDYSSYVGAIGIGRIQRGTIRRNTPVIII        240
      Scurry Q217     TDMTPLFETIVSKVAPPQVDLNGPFQMQISSLDYSSYVGAIGIGRIQRGTIRRNTPVIII        240
            Q229      TDMTPLFETIVSKVAPPQVDLNGPFQMQISSLDYSSYVGAIGIGRIQRGTIRRNTPVIII        240
         CbuG Q212    TDMTPLFETIVSKVAPPQVDLNGPFQMQISSLDYSSYVGAIGIGRIQRGTIRRNTPVIII        240
  Dugway 5J108-111    TDMTPLFETIVSKVAPPQVDLNGPFQMQISSLDYSSYVGAIGIGRIQRGTIRRNTPVIII        240
          Namibia     TDMTPLFETIVSKVAPPQVDLNGPFQMQISSLDYSSYVGAIGIGRIQRGKIRRNTPVIII        240
         CbuK Q154    TDMTPLFETIVSKVAPPQVDLNGPFQMQISSLDYSSYVGAIGIGRIQRGTIRRNTPVIII        240
   MSU 'Goat' Q177    TDMTPLFETIVSKVAPPQVDLNGPFQMQISSLDYSSYVGAIGIGRIQRGTIRRNTPVIII        240
            Q321      TDMTPLFETIVSKVAPPQVDLNGPFQMQISSLDYSSYVGAIGIGRIQRGTIRRNTPVIII        240
                      **************.**********************************.**********

          RSA331      DREGKRRSGRVLQLLGFLGLQRVDIETAEAGDIVAVTGIENLRISDTLCDPQQVEALPPL        300
       RSA493 NMI     DREGKRRSGRVLQLLGFLGLQRVDIETAEAGDIVAVTGIENLRISDTLCDPQQVEALPPL        300
      Scurry Q217     DREGKRRSGRVLQLLGFLGLQRVDIETAEAGDIVAVTGIENLRISDTLCDPQQVEALPPL        300
            Q229      DREGKRRSGRVLQLLGFLGLQRVDIETAEAGDIVAVTGIENLRISDTLCDPQQVEALPPL        300
         CbuG Q212    DREGKRRSGRVLQLLGFLGLQRVDIETAEAGDIVAVTGIENLRISDTLCDPQQVEALPPL        300
  Dugway 5J108-111    DREGKRRSGRVLQLLGFLGLQRVDIETAEAGDIVAVTGIENLRISDTLCDPQQVEALPPL        300
          Namibia     DREGKRRSGRVLQLLGFLGLQRVDIETAEAGDIVAVTGIENLRISDTLCDPQQVEALPPL        300
         CbuK Q154    DREGKRRSGRVLQLLGFLGLQRVDIETAEAGDIVAVTGIENLRISDTLCDPQQVEALPPL        300
   MSU 'Goat' Q177    DREGKRRSGRVLQLLGFLGLQRVDIETAEAGDIVAVTGIENLRISDTLCDPQQVEALPPL        300
            Q321      DREGKRRSGRVLQLLGFLGLQRVDIETAEAGDIVAVTGIENLRISDTLCDPQQVEALPPL        300
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          RSA331      TVDEPTVSMTFQVNNSPFAGREGKFLTSRQIKERLEKELIANVALRVAAGADADKFIVSG        360
       RSA493 NMI     TVDEPTVSMTFQVNNSPFAGREGKFLTSRQIKERLEKELIANVALRVAAGADADKFIVSG        360
      Scurry Q217     TVDEPTVSMTFQVNNSPFAGREGKFLTSRQIKERLEKELIANVALRVAAGADADKFIVSG        360
            Q229      TVDEPTVSMTFQVNNSPFAGREGKFLTSRQIKERLEKELIANVALRVAAGADADKFIVSG        360
         CbuG Q212    TVDEPTVSMTFQVNNSPFAGREGKFLTSRQIKERLEKELIANVALRVAAGADADKFIVSG        360
  Dugway 5J108-111    TVDEPTVSMTFQVNNSPFAGREGKFLTSRQIKERLEKELIANVALRVAAGADADKFIVSG        360
          Namibia     TVDEPTVSMTFQVNNSPFAGREGKFLTSRQIKERLEKELIANVALRVAAGADADKFIVSG        360
         CbuK Q154    TVDEPTVSMTFQVNNSPFAGREGKFLTSRQIKERLEKELIANVALRVAAGADADKFIVSG        360
   MSU 'Goat' Q177    TVDEPTVSMTFQVNNSPFAGREGKFLTSRQIKERLEKELIANVALRVAAGADADKFIVSG        360
            Q321      TVDEPTVSMTFQVNNSPFAGREGKFLTSRQIKERLEKELIANVALRVAAGADADKFIVSG        360
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          RSA331      RGELHLSILIENMRREGYEFSVSRPEVIKKMVDDIECEPFENLVLDIDEEHQGDIIQNLA        420
       RSA493 NMI     RGELHLSILIENMRREGYEFSVSRPEVIKKMVDDIECEPFENLVLDIDEEHQGDIIQNLA        420
      Scurry Q217     RGELHLSILIENMRREGYEFAVSRPEVIKKMVDDIECEPFENLVLDIDEEHQGDIIQNLA        420
            Q229      RGELHLSILIENMRREGYEFAVSRPEVIKKMVDDIECEPFENLVLDIDEEHQGDIIQNLA        420
         CbuG Q212    RGELHLSILIENMRREGYEFAVSRPEVIKKMVDDIECEPFENLVLDIDEEHQGDIIQNLA        420
  Dugway 5J108-111    RGELHLSILIENMRREGYEFAVSRPEVIKKMVDDIECEPFENLVLDIDEEHQGDIIQNLA        420
          Namibia     RGELHLSILIENMRREGYEFAVSRPEVIKKMVDDIECEPFENLVLDIDEEHQGDIIQNLA        420
         CbuK Q154    RGELHLSILIENMRREGYEFAVSRPEVIKKMVDDIECEPFVNLVLDIDEEHQGDIIQNLA        420
   MSU 'Goat' Q177    RGELHLSILIENMRREGYEFAVSRPEVIKKMVDDIECEPFVNLVLDIDEEHQGDIIQNLA        420
            Q321      RGELHLSILIENMRREGYEFAVSRPEVIKKMVDDIECEPFENLVLDIDEEHQGDIIQNLA        420
                      ********************:********************* ********************

          RSA331      KRKGDLKNMMPGGKGRVRLDYLIPTRGLIGFHSHFLTLTSGSGVMYHVFDHYAPLIEESL        480
       RSA493 NMI     KRKGDLKNMMPGGKGRVRLDYLIPTRGLIGFHSHFLTLTSGSGVMYHVFDHYAPLIEESL        480
      Scurry Q217     KRKGDLKNMMPGGKGRVRLDYLIPTRGLIGFHSHFLTLTSGSGVMYHVFDHYAPLIEESL        480
            Q229      KRKGDLKNMMPGGKGRVRLDYLIPTRGLIGFHSHFLTLTSGSGVMYHVFDHYAPLIEESL        480
         CbuG Q212    KRKGDLKNMMPGGKGRVRLDYLIPTRGLIGFHSHFLTLTSGSGVMYHVFDHYAPLIEESL        480
  Dugway 5J108-111    KRKGDLKNMMPDGKGRVRLDYLIPTRGLIGFHSHFLTLTSGSGVMYHVFDHYAPLIEESL        480
          Namibia     KRKGDLKNMMPDGKGRVRLDYLIPTRGLIGFHSHFLTLTSGSGVMYHVFDHYAPLIEESL        480
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      CbuK Q154      KRKGDLKNMMPDGKGRVRLDYLIPTRGLIGFHSHFLTLTSGSGVMYHVFDHYAPLIEESL        480
MSU 'Goat' Q177      KRKGDLKNMMPDGKGRVRLDYLIPTRGLIGFHSHFLTLTSGSGVMYHVFDHYAPLIEESL        480
           Q321      KRKGDLKNMMPDGKGRVRLDYLIPTRGLIGFHSHFLTLTSGSGVMYHVFDHYAPLIEESL        480
                     ***********.**************************************************

         RSA331      QTRHRGVLISNSQGVATAYALWNLQSRGNLFIGPQQAVYEGMIVGQHSRDNDLVVNVCRE        540
      RSA493 NMI     QTRHRGVLISNSQGVATAYALWNLQSRGNLFIGPQQAVYEGMIVGQHSRDNDLVVNVCRE        540
     Scurry Q217      QTRHRGVLISNSQGVATAYALWNLQSRGNLFIGPQQAVYEGMIVGQHSRDNDLVVNVCRE        540
           Q229      QTRHRGVLISNSQGVATAYALWNLQSRGNLFIGPQQAVYEGMIVGQHSRDNDLVVNVCRE        540
       CbuG Q212     QTRHRGVLISNSQGVATAYALWNLQSRGNLFIGPQQAVYEGMIVGQHSRDNDLVVNVCRE        540
Dugway 5J108-111     QTRHRGVLISNSQGVATAYALWNLQSRGNLFIGPQQAVYEGMIVGQHSRDNDLVVNVCRE        540
         Namibia     QTRHRGVLISNSQGVATAYALWNLQSRGNLFIGPQQAVYEGIIVGQHSRDNDLVVNVCRE        540
       CbuK Q154     QTRHRGVLISNSQGVATAYALWNLQSRGNLFIGPQQAVYEGIIVGQHSRDNDLVVNVCRE        540
MSU 'Goat' Q177      QTRHRGVLISNSQGVATAYALWNLQSRGNLFIGPQQAVYEGIIVGQHSRDNDLVVNVCRE        540
           Q321      QTRHRGVLISNSQGVATAYALWNLQSRGNLFIGPQQAVYEGIIVGQHSRDNDLVVNVCRE        540
                     ****************************************:*********************

         RSA331      KQLTNIRAAGSDENIILTPPIKFSLEQALQFIADDELVEITPAAIRLRKKLLKEHERRRA        600
      RSA493 NMI     KQLTNIRAAGSDENIILTPPIKFSLEQALQFIADDELVEITPAAIRLRKKLLKEHERRRA        600
     Scurry Q217      KQLTNIRAAGSDENIILTPPIKFSLEQALQFIADDELVEITPAAIRLRKKLLKEHERRRA        600
           Q229      KQLTNIRAAGSDENIILTPPIKFSLEQALQFIADDELVEITPAAIRLRKKLLKEHERRRA        600
       CbuG Q212     KQLTNIRAAGSDENIILTPPIKFSLEQALQFIADDELVEITPAAIRLRKKLLKEHERRRA        600
Dugway 5J108-111     KQLTNIRAAGSDENIILTPPIKFSLEQALQFIADDELVEITPAAIRLRKKLLKEHERRRA        600
         Namibia     KQLTNIRAAGSDENIILTPPIKFSLEQALQFIADDELVEITPAAIRLRKKLLKEHERRRA        600
       CbuK Q154     KQLTNIRAAGSDENIILTPPIKFSLEQALQFIADDELVEITPAAIRLRKKLLKEHERRRA        600
MSU 'Goat' Q177      KQLTNIRAAGSDENIILTPPIKFSLEQALQFIADDELVEITPAAIRLRKKLLKEHERRRA        600
           Q321      KQLTNIRAAGSDENIILTPPIKFSLEQALQFIADDELVEITPAAIRLRKKLLKEHERRRA        600
                     ************************************************************

         RSA331      ER        602
      RSA493 NMI     ER        602
     Scurry Q217      ER        602
           Q229      ER        602
       CbuG Q212     ER        602
Dugway 5J108-111     ER        602
         Namibia     ER        602
       CbuK Q154     ER        602
MSU 'Goat' Q177      ER        602
           Q321      ER        602
                     **
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Figure 3-10. Alignment of the TypA GTP-binding protein from the ten *C. burnetii* strains. * indicates identical residues, : and . indicate conserved and weakly conserved residues.

strains are equally divided at position D432G, with RSA331, NMI, Scurry Q217, and CbuG Q212 all with the G432 allele and the rest with the D432 allele. Two conserved amino acid variations were observed in proteins from strains RSA331 and NMI (A381S) and in proteins from strains Namibia, CbuK Q154, MSU 'Goat' Q177 and Q321 (M522I). Only one unconserved amino acid variation was observed in strains CbuK Q154 and MSU 'Goat' Q177 at position E401V.

The program Modeller was used to predict structures for these putative antibacterial susceptibility proteins based on homology to the amino acid sequences with solved crystal structures and visualized using Chimera (Petterson et al., 2004; Webb and Sali, 2016). These allowed for the comparison of structures between *C. burnetii* isolates to determine similarities and differences among the groups of isolates. The overlaying of predicted structures from different clustered did not reveal any striking anomalies in the comparisons (data not shown). This however, does identify a deficiency of solved protein structures for *C. burnetii* that may be a valuable resource to future development of therapeutic targets with which Q fever can be treated.

Collectively these results suggest that the origins of putative antibacterial susceptibility genes among the *C. burnetii* strains analyzed in this study vary depending upon the strains and highlight the genetic diversity of putative antibacterial susceptibility genes among the strains for certain classes of antimicrobial agents. This informatic review of putative antibacterial susceptibility genes also demonstrates the value of identifying the presence of those genes in cultures from patients with Q fever to tailor antibacterial

treatments based upon the susceptibility profile of the strains present. The developed culture requirements for *C. burnetii* are specialized, require designated BSL-3 laboratory safety measures, are likely unavailable in most clinical diagnostic laboratories. Therefore it is crucial to further the foundational knowledge of how antibiotics are affected by the acidic environment in which *C. burnetii* propagates inside host cells and how antibiotics can be screened both in liquid and solidified medium.

Effects of acidic growth conditions on MIC of ampicillin treated E. coli cultures

In optimal intracellular and axenic growth conditions, 37°C, 5% CO_2, and 2.5% O_2, pH 4.75) *C. burnetii* NMI and NMII cultures have a doubling time of approximately sixteen hours (Sandoz *et al.*, 2016). This combination of a relatively slow replication rate and acidic environment is suspected to have a considerable impact upon the efficacy of antibiotics in the treatment of Q fever. However, there is a glaring gap in the literature that has explored this topic, which this study was designed to begin addressing. Given that functionality of antimicrobial agents is intrinsically tied to their structures, it was predicted that antimicrobial efficacy will be greatly hindered by sustained exposure to the low pH environment suitable for *C. burnetii* growth. In order to quickly ascertain the effects of pH on antibiotics on cultures, the 1X ACCM-D liquid broth was used untreated with antibiotics, pre-treated with antibiotics, and treated at time of inoculation with *E. coli* in 96-well culture plates to quickly measure bacterial growth by optical density in a shaking, plate reader heated to 37°C. This strategy allowed us to use a standard laboratory bacterial workhorse of *E. coli* with well characterized growth kinetics and MICs for a broad spectrum of antibiotics to focus on the effects of pH 4.75

has on antibiotic efficacy. A broad library of bacterial species was screened prior to this test by inoculating colonies of bacteria into 1X ACCM-D pH 4.75 at 37°C 5% CO_2, 2.5% O_2, without agitation, and observed which strains were able to grow as does *C. burnetii*. Of the many bacteria that grew, *E. coli* was selected as the best characterized with robust growth under those conditions (data not shown). Since the plate reader used for these studies had no function to regulate levels of CO_2 and O_2, experiments were carried out under atmospheric conditions.

Trials were conducted with 1X ACCM-D pH 4.75 and 1X ACCM-D pH 7.0 to compare the differences in antibiotic efficacy during aerobic growth of *E. coli*. Overnight cultures of *E. coli* were growth in either 1X ACCM-D pH 4.75 or 1X ACCM-D pH 7.0, and diluted 1:100 and grown until same OD_{600} is reached (equivalent to $1x10^8$ cells/mL) prior to inoculating $5x10^6$ cells/well in 96-well flat-bottom culture plates with 200 μL/well 1X ACCM-D pH 4.75 or 1X ACCM-D pH 7.0 and bacterial growth was monitored OD_{600} in a plate reader heated to 37°C with shaking of the plate every 15 seconds and absorbance read every minute. Wells had medium untreated with antibiotics as a control, pre-treated by the addition of antibiotics one day prior to inoculation, pre-treated two days by the addition of antibiotic prior to inoculation, or treated at the time of inoculation to assess how the pH of the medium affected inhibitory effects of dilution series of ampicillin (Gold Biotechnologies, USA), chloramphenicol (Gold Biotechnologies, USA) or tetracycline (Millapore Sigma, USA). Antibiotic stocks were generated based on concentration of the particular lots of the antibiotics according to the CLSI guidelines (CLSI, 2012). Traditional MIC assays use overnight incubations of bacteria grown on

solidified growth media supplemented with antibiotics. The kinetic growth of the bacteria in liquid cultures was ideal for this observations to gain more insights into how pH affects bacterial growth inhibition in the presence of antibiotics exposed to different pH environments. The MICs reported below merely reflect the inhibition of growth following five hours of growth in liquid culture. Results from the ampicillin trials comparing *E. coli* growth in 1X ACCM-D pH 4.75 and 1X ACCM-D pH 7.0 with cultures pre-treated with ampicillin two days prior to inoculation, one day prior to inoculation, treated at time of inoculation, or untreated revealed a difference in growth kinetics between the low and neutral pH growth conditions (Figure 3-11 A-C and Figure 3-11 D-F, respectively). Analysis of the data revealed that the MIC of ampicillin for cultures grown at both pH 4.75 and pH 7.0 was between 25 and 50 μg/mL for all treatment times. However, after 75 minutes, inhibition of bacterial growth is observed under neutral pH conditions for cultures growing in the presence of 25 μg/mL ampicillin.

These trials with *E. coli* were conducted again with a dilution series of the more clinically relevant tetracycline antibiotic under the same conditions as for ampicillin (Figure 3-11). Differences were observed in growth kinetics when treating cultures with a bacteriostatic antibiotic, compared to the bactericidal ampicillin. Differences were observed in the bacterial growth rates between pH 4.75 and pH 7.0 conditions (Figure 3-12 A-C and Figure 3-12 D-F, respectively). Analysis of the data reveal that the MIC of tetracycline for cultures grown at pH 4.75 is 16 μg/mL compared to the MIC of 4 μg/mL for cultures grown at pH 7.0 for all treatment times tested. A four-fold increase in the

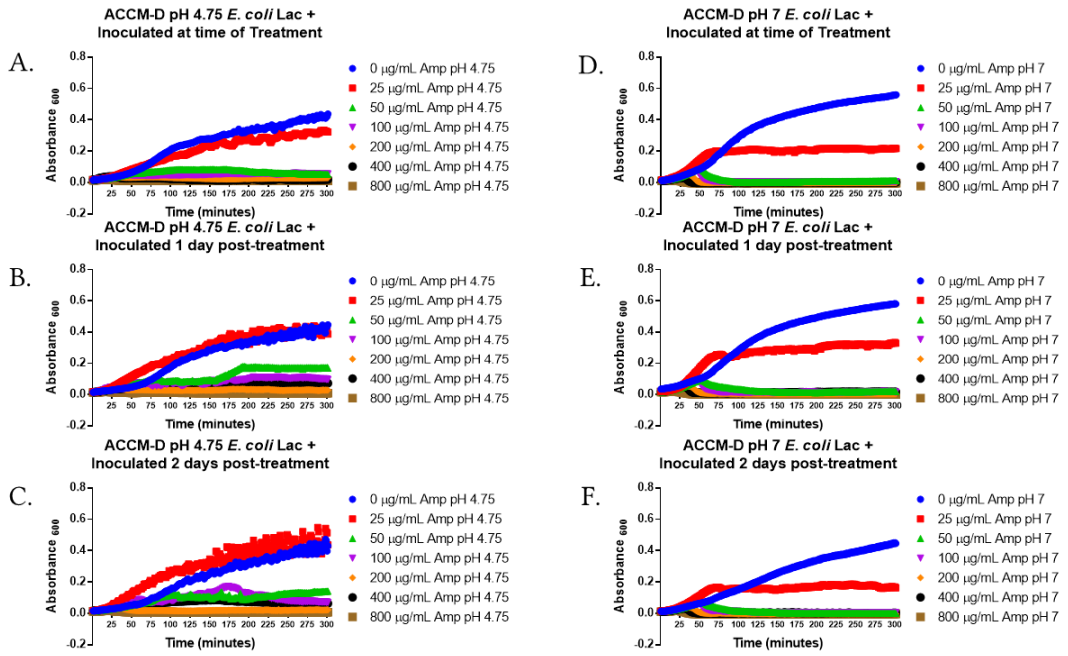

Figure 3-11. Effects of 1X ACCM-D pH 4.75 and 1X ACCM-D pH 7.0 on ampicillin MICs for *E. coli* growth over different exposure times. A. Growth of *E. coli* inoculated into 1X ACCM-D pH 4.75 at the time the medium was treated with a dilution series of ampicillin concentrations. B. Growth of *E. coli* inoculated into 1X ACCM-D pH 4.75 one day after the medium was treated with a dilution series of ampicillin concentrations. C. Growth of *E. coli* inoculated into 1X ACCM-D pH 4.75 two days after the medium was treated with a dilution series of ampicillin concentrations. D. Growth of *E. coli* inoculated into 1X ACCM-D pH 7.0 at the time the medium was treated with a dilution series of ampicillin concentrations. E. Growth of *E. coli* inoculated into 1X ACCM-D pH 7.0 one day after the medium was treated with a dilution series of ampicillin concentrations. F. Growth of *E. coli* inoculated into 1X ACCM-D pH 7.0 two days after the medium was treated with a dilution series of ampicillin concentrations.

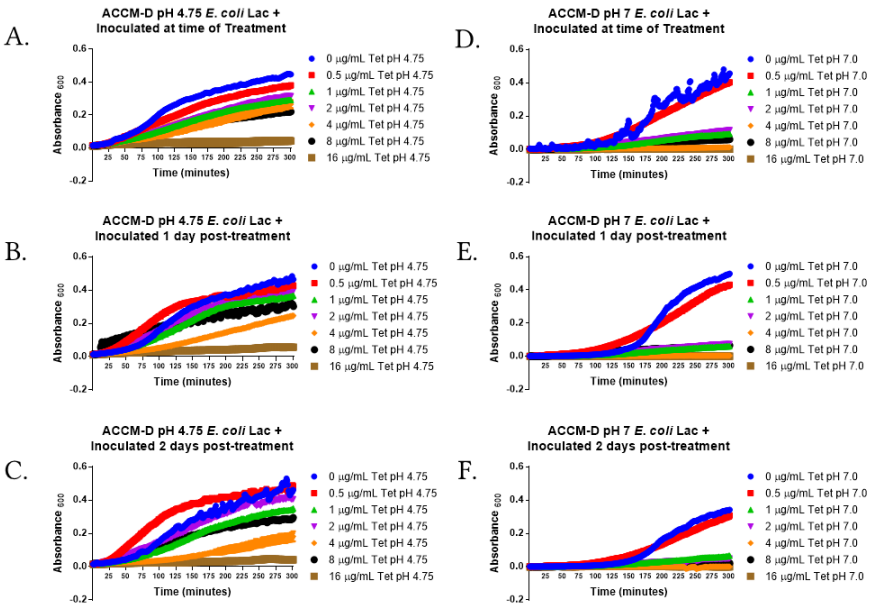

Figure 3-12. Effects of 1X ACCM-D pH 4.75 and 1X ACCM-D pH 7.0 on tetracycline MICs for *E. coli* growth over different exposure times. A. Growth of *E. coli* inoculated into 1X ACCM-D pH 4.75 at the time the medium was treated with a dilution series of tetracycline concentrations. B. Growth of *E. coli* inoculated into 1X ACCM-D pH 4.75 one day after the medium was treated with a dilution series of tetracycline concentrations. C. Growth of *E. coli* inoculated into 1X ACCM-D pH 4.75 two days after the medium was treated with a dilution series of tetracycline concentrations. D. Growth of *E. coli* inoculated into 1X ACCM-D pH 7.0 at the time the medium was treated with a dilution series of tetracycline concentrations. E. Growth of *E. coli* inoculated into 1X ACCM-D pH 7.0 one day after the medium was treated with a dilution series of tetracycline concentrations. F. Growth of *E. coli* inoculated into 1X ACCM-D pH 7.0 two days after the medium was treated with a dilution series of tetracycline concentrations.

MIC of tetracycline under the acidic growth conditions in which *C. burnetii* is treated is a striking observation that highlights the importance of better understanding factors that contribute to *C. burnetii* persistence in host cells.

Lastly, results from the chloramphenicol trials comparing *E. coli* growth in 1X ACCM-D pH 4.75 and 1X ACCM-D pH 7.0 with cultures pre-treated with chloramphenicol two days prior to inoculation, one day prior to inoculation, treated at time of inoculation, or without antibiotics reveal differences in growth kinetics between the low and neutral pH growth conditions (Figure 3-13 A-C and Figure 3-13 D-F, respectively). Analysis of the data reveal that the MIC of chloramphenicol for cultures grown at pH 4.75 and at pH 7.0 was 16 μg/mL. Length of treatment of the medium with antibiotics has little effect on the bacterial growth at pH 4.75. In contrast, bacterial growth appears to decrease when the time the medium is treated with antibiotics increases.

Cumulatively, these data highlight the inhibitory role of a low pH environment on antibiotic efficacy and provide valuable insights into the pitfalls of running an antibiotic treatment time course in *C. burnetii* axenic medium that would last one to two weeks given traditional culture methods. Exploration of alternative culture methods to obtain a snap shot of how antibiotics affect *C. burnetii* growth in axenic medium is needed to broaden the understanding of antibiotic susceptibility in *C. burnetii* axenic cultures.

Development of methods to assess inhibitory effects of antibiotics on virulent C. burnetii

Having gained an appreciation for the effects of pH on antibiotic efficacy, next, how the

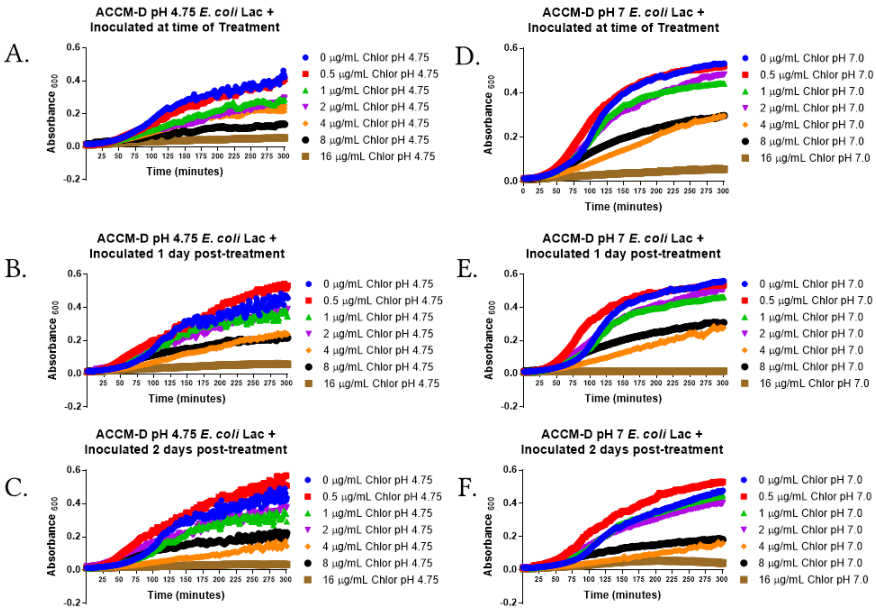

Figure 3-13. Effects of 1X ACCM-D pH 4.75 and 1X ACCM-D pH 7.0 on chloramphenicol (chlor) MICs for *E. coli* growth over different exposure times. A. Growth of *E. coli* inoculated into 1X ACCM-D pH 4.75 at the time the media was treated with a dilution series of chlor concentrations. B. Growth of *E. coli* inoculated into 1X ACCM-D pH 4.75 one day after the media was treated with a dilution series of chlor concentrations. C. Growth of *E. coli* inoculated into 1X ACCM-D pH 4.75 two days after the media was treated with a dilution series of chlor concentrations. C. Growth of *E. coli* inoculated into 1X ACCM-D pH 7.0 at the time the media was treated with a dilution series of chlor concentrations. E. Growth of *E. coli* inoculated into 1X ACCM-D pH 7.0 one day after the media was treated with a dilution series of chlor concentrations. F. Growth of *E. coli* inoculated into 1X ACCM-D pH 7.0 two days after the media was treated with a dilution series of chlor concentrations.

axenic medium, 1X ACCM-D at pH 4.75, can best be utilized to assay antibiotic susceptibility on virulent strains of *C. burnetii* cultured at 37°C 5% CO_2, 2.5% O_2 without agitation, as is typical to culture *C. burnetii* strains was explored.

Culture practices for *C. burnetii* present challenges in terms of establishing quantifiable bacterial numbers used in assays. Cultures of *E. coli* can be quantified by optical density to infer bacterial numbers and can be plated on agar to enumerate CFUs in the culture within sixteen hours of incubation. Established practices for enumerating cultures or freezer stocks of *C. burnetii* were developed prior to the advent of axenic medium and therefore relied upon genome equivalents of *com1* gene numbers present in the culture (Brennan and Samuel, 2003). Optical measurements of changes in turbidity commonly used in other bacteria currently have little practical use measuring growth of *C. burnetii* in axenic medium where a 10-day culture yields a maximum turbidity less than 0.06 at OD_{600}. Use of solidified ACCM-D allows for the plating and enumeration of colonies to determine CFU numbers for stocks, however, this presented a discrepancy. Genome equivalents do not discriminate between viable and non-viable bacteria, whereas, CFU only represent viable bacteria. Moreover, some virulent strain freezer stocks displayed higher sensitivity to repeated freeze-thaw, yielding discrepancies of between one order of magnitude and three orders of magnitude when comparing the titer determined by genome equivalents and titers determined by CFUs. Given these challenges, the data presented here are from assays with genome equivalents to calculate approximate equal inoculum to add to medium, readers will observe a range of CFUs at the zero time points, resulting from the discrepancies mentioned above.

An additional impetus to explore methods alternative to determining MICs as genome equivalents (GEs) stems from the material and time intensive costs of performing real-time PCR on dilution series in replicates for multiple strains and multiple antibiotics.

Since the objective of this portion of the project was to generate methods to facilitate the determination of MICs in axenic cultures of *C. burnetii*, 96-well plates were used to culture strains of *C. burnetii* by placing the stationary plates in the same incubator used to culture *C. burnetii* in stationary flasks at 37°C 5% CO_2, 2.5% O_2. Protocol validation began with the avirulent *C. burnetii* NMII diluted to 5.5 x 10^4 GE/mL in 1X ACCM-2 pH 4.75 with 0.1 mL used as inoculum for each well containing 0.1 mL of 1X ACCM-2 pH 4.75 that was untreated, treated with a dilution series of ampicillin or streptomycin (25 μg/mL, 50 μg/mL, 100 μg/mL). To minimize risk of evaporation of culture media, samples were surrounded by wells of sterile water. The 96-well plate containing the cultures were incubated at 37°C 5% CO_2, 2.5% O_2 for 4 days. On day four, cultures were diluted 10-fold in 1X ACCM-2 and 5 μL of each dilution was spotted on tilted solid agarose 1X ACCM-2 plates to allow the spots to elongate into columns and incubated at 37°C 5% CO_2, 2.5% O_2 for 14 days and colonies were counted to determine CFU/mL (Figure 3-14). Samples from the dilution series were also processed for genome equivalents by real-time PCR to ascertain the genomic copies present in the dilutions. Comparison of the CFU/mL and GE/mL reveal an approximately 2 order of magnitude decrease of viable cells compared to genomic copy values from untreated cultures

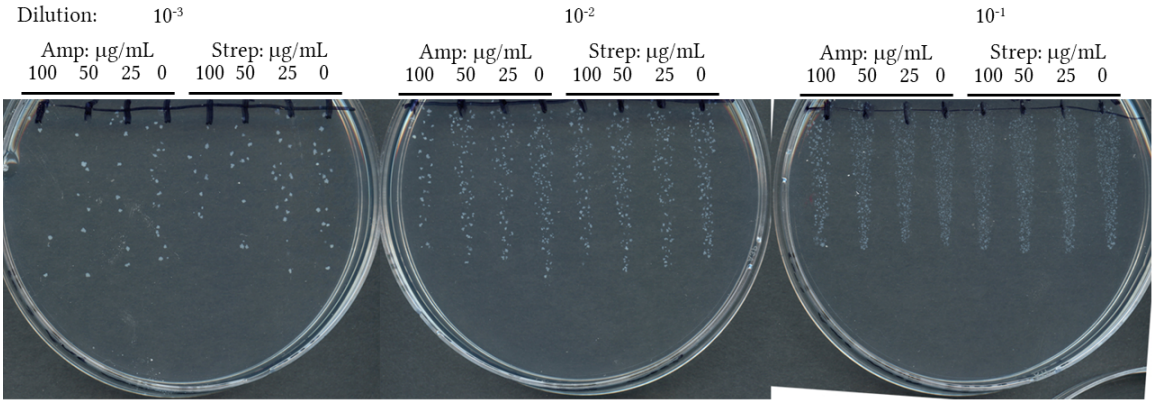

Figure 3-14. Example of spot plating method of 10-fold dilution series of 96-well *C. burnetii* NMII cultures. Four day cultures were diluted 10-fold in 1X ACCM-2 and 5 µL of each dilution was spotted on tilted solid agarose 1X ACCM-2 plates to allow the spots to elongate into columns and incubated at 37°C 5% CO_2, 2.5% O_2 for 14 days for colonies to develop.

(Figure 3-15). Despite the difference in bacterial titers, there is a high degree of agreement between the two methods of bacterial enumeration. An important observation is the high level of resistance to ampicillin exhibited by NMII, this suggests higher concentrations of antibiotics will be required to observe inhibition of bacterial growth. These results validate that this approach is a practical method to quantify bacterial numbers of cultures treated with antibiotics.

Translation of these methods to the virulent *C. burnetii* strains identified additional challenges beyond performing the methods under BSL-3 conditions; such as differences in GE/mL to CFU/mL ratios, differences in colony morphology, and differences in viability of bacterial freezer stocks after repeated freeze-thaw. Initial validation of this protocol on virulent strains of *C. burnetii* focused on ampicillin as the homology of the proteins was high (99% amino identity, 99% amino positives). A panel of virulent strains was selected representing different sources of isolation. The strain NMI RSA 493 was isolated from a tick in the U.S.A. in 1935; Henzerling RSA 331 was isolated from human blood in Italy in 1945; Q229 was isolated from a human's infected heart valve in Nova Scotia in 1982; and Scurry Q217 was isolated from human liver biopsy in the U.S.A. in 1981 (Zhang *et al.*, 2005). Each strain had been passaged twice in 1X ACCM-D pH 4.75 to acclimate the strains to axenic growth conditions. Ampicillin concentrations used in the 96-well cultures were 25 μg/mL, 50 μg/mL, 100 μg/mL, 200 μg/mL, 400 μg/mL, 800 μg/mL, and control wells contained no ampicillin. Genome equivalents were used to calculate dilution of freezer stocks in 1X ACCM-D pH 4.75 to seed each well at $5x10^6$ GE

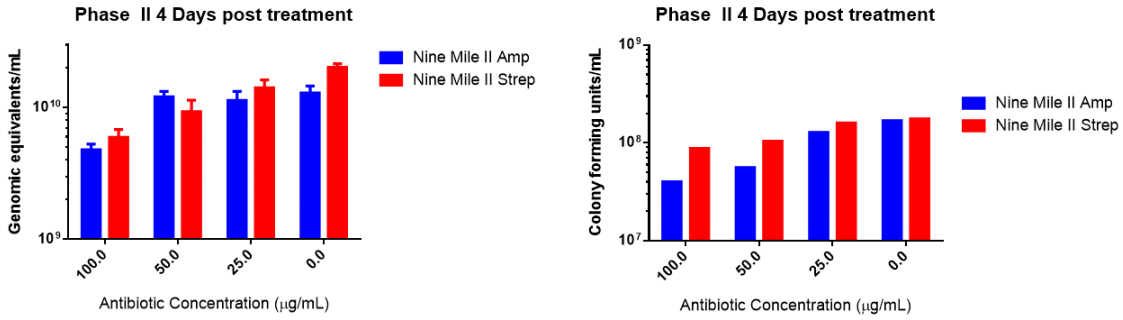

Figure 3-15. Comparison of genome equivalencies to colony forming units of NMII with and without ampicillin treatment at four days of growth. Enumeration of bacterial numbers from real-time PCR and CFU from four day cultures in 1X ACCM-2 incubated at 37°C 5% CO_2, 2.5% O_2 and processed for genomic DNA isolation and diluted for plating.

in 0.1 mL per well.

Dilutions of antibiotics were added to each well at 0.1 mL to reach the final concentrations listed above and cultures were incubated at 37°C 5% CO_2, 2.5% O_2 for 2 days to allow for three rounds of replication. Dilutions of the seeding cultures were plated onto solid agarose 1X ACCM-D plates to determine the viable bacterial CFU to compare to the genome equivalents of the stocks. The dilutions of the seed stocks were incubated at 37°C 5% CO_2, 2.5% O_2 for 14 days for colonies to develop. After two days, cultures from the 96-well plate were diluted and plated onto solid agarose 1X ACCM-D plates to determine the viable bacterial CFU and incubated at 37°C 5% CO_2, 2.5% O_2 for 14 days for colonies to develop. Bacterial plates of the seed stocks calculated at $5x10^6$ GE/well, revealed that the viable bacterial counts at the time of inoculation were $6x10^5$ CFU/well, $9.2x10^5$ CFU/well, $2.9x10^5$ CFU/well, and $9x10^5$ CFU/well for NMI, Q217, Q229, and RSA 331, respectively. This follows the observed trend where viable counts are approximately one order of magnitude lower than the genome equivalents. Results from the cultures grown with and without ampicillin treatment after two days of incubation reveals a remarkable array of susceptibility phenotypes for each of the four strains (Figure 3-16). The virulent strain Henzerling RSA 331 exhibits complete resistance to ampicillin concentrations used prior to plating. Strains Q217 and NMI exhibit similar levels of bacterial numbers following ampicillin treatment and reveals inhibited bacterial numbers following exposure to ampicillin at concentrations of 100 μg/mL, 200 μg/mL. Virulent strain Q229 also displayed inhibited bacterial growth

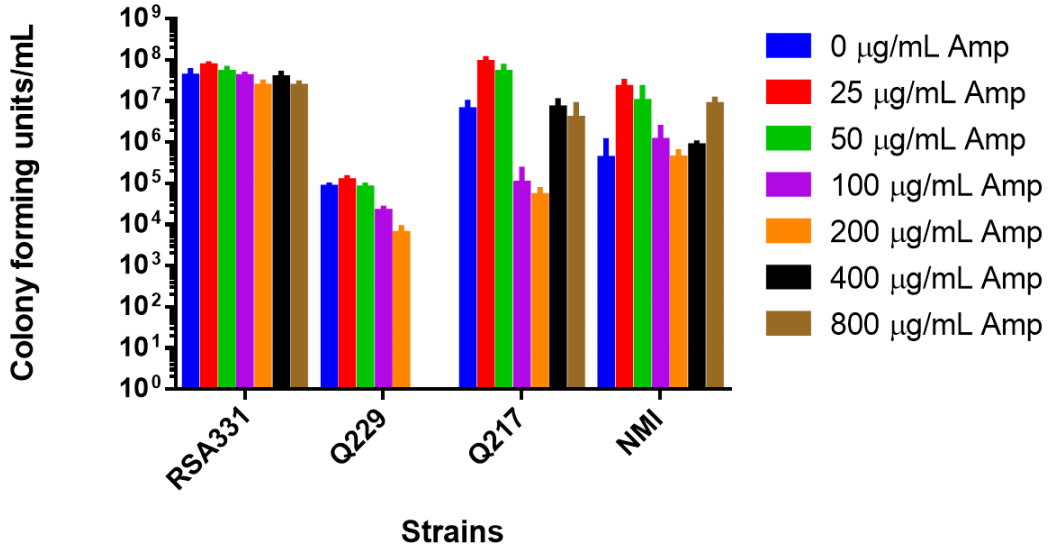

Figure 3-16. Viable bacterial numbers following ampicillin treatment over 2 days reveals differences of inhibition to bacterial growth among virulent strains. All strains, except RSA 331, exhibit inhibited growth after treatment with 100 μg/mL and 200 μg/mL of ampicillin.

following exposure to ampicillin at concentrations of 100 μg/mL, 200 μg/mL, however no colonies were found in the dilutions from wells treated with 400 μg/mL and 800 μg/mL of ampicillin. From these initial trials it remains unclear why treatment with 400 μg/mL and 800 μg/mL ampicillin for two days resulted in less inhibited bacterial growth, specifically in strains Q217 and NMI. *C. burnetii* has a well documented resistance to environmental stressors in the small cell variant, which is likely to form after 14 days growth of the stocks in 1X ACCM-D (Babudieri, 1959; McCaul *et al.*, 1981). Additional factors may be involved in this slow-growing bacterium, such as the antibiotic tolerant forms of *M. tuberculosis* that exhibit metabolic activity, yet do not grow until environmental stressors are removed (Manina *et al.*, 2015).

The development of these methods illustrate the utility and practicality of their use in determining viable bacterial numbers and as a method to continue the exploration of how antibiotics affect *C. burnetii* growth in axenic media. Further development and studies are required to ascertain what time points are most informative to indicate inhibitory growth. While *C. burnetii* growth in axenic media is a slow process, taking up to two weeks for colony development, the methods established in this study offer a quicker and cheaper alternative to real-time PCR analysis that requires isolation of bacterial DNA and cannot discriminate between non-viable and viable bacterial numbers.

Discussion

This study marks an important venture into better understanding *C. burnetii* antibiotic susceptibility by analyzing genomes of eight *C. burnetii* strains for the presence of genes with putative roles in antibiotic susceptibility. While there have been detailed studies characterizing the properties of the CCV and establishing its pH of 4.75 and speculation of how this may affect function of antibiotics, to date, this is the first study to demonstrate the reduction of antibiotic efficacy that is experienced while antibiotics are in this environment (Hackstadt and Williams, 1981; Hackstadt, 1990; Raoult *et al.*, 1990; Maurin and Raoult, 1999). The development of axenic media to propagate *C. burnetii* outside the host has been a critical advancement in the field, yet the initial cumbersome published methods for plating are not suitable for adopting standard methods in antibiotic susceptibility testing (Omsland *et al.*, 2011; Beare and Heinzen, 2014; Martinez *et al.*, 2015). After adopting the new axenic media culturing system, alterations were made that enhanced the versatility of the solid cultures by eliminating the use of ACCM semi-solid overlays. This not only facilitated media preparation efforts by simplifying the process, but also allowed for use of multi-channel pipettes to spot entire dilution series onto a single plate, vastly reducing materials and time needed for bacterial survival plating from cell culture and infected animal tissues and MIC plating. Additionally, elimination of the semi-solid overlay allowed trials to explore the feasibility of disk diffusion of antibiotics, known as the Kirby-Bauer disk diffusion susceptibility test. Removal of the overlay also greatly streamlined the process for selecting colonies of mutants. Finally, the development of axenic medium and auxotrophic cloning and knock-out vectors have led to an explosion of much needed understanding of *C. burnetii* genetics, which largely has focused on T4BSS effectors,

factors in metabolism, and virulence related factors (Clemente *et al.*, 2022; Cheng *et al.*, 2022; Zhang *et al.*, 2022). However, this study marks the first steps to begin characterizing genes with putative involvement in antibiotic susceptibility outside the host.

These results lay the foundation for the challenging process of creating a widely accepted axenic medium-based approach for the determination of MIC of antibiotics. The trials with virulent strains reveal numerous challenges, including loss in viability following freeze-thaw of stocks, discrepancies between GE/mL and CFU/mL, and differences in colony morphology. However, the results also highlight the cost- and time-saving benefits of adopting the axenic medium-based approach even if only applicable to research laboratories wishing to compare the inhibitory effects of antibiotics among virulent strains. Additional studies are required to explore the effects of the putative antibiotic susceptibility proteins identified in this study against the full panel of antibiotics to identify alleles linked to antibiotic susceptibility that can be further examined by cloning into *E. coli* and ultimately using *C. burnetii* auxotrophic cloning vectors to delete those genes in virulent strains. These future studies are critical to filling the gap in knowledge of factors that influence antibiotic susceptibility in *C. burnetii* strains and could advance PCR-based screening methods to identify antibiotic susceptibility alleles that may influence treatment methods in patients afflicted with Q fever.

Future directions combined with the next generation sequencing (NGS) technology of

RNA-Seq and LC-MS/MS to identify the transcripts and proteins involved in antibiotic resistance and probe the connections between acute and chronic infections in relation to antibiotic resistance at the molecular level.

Experimental Procedures

Bacterial strains and cultures

Isolates of *C. burnetii* (Scurry Q217, Ko-Q229, Henzerling RSA 331, RSA 493 Nine Mile I were obtained from Dr. James E. Samuel at Texas A&M University. Stocks used in this study were cultured in 1X ACCM-D with initial pH of 4.75 and passaged twice in 1X ACCM-D before freezer stocks were made. The isolate *E. coli* K-12 substr. MG1655 was kindly provided by Dr. Heidari B. Manijeh of the Department of Molecular Microbiology & Immunology at the School of Medicine at the University of Missouri – Columbia.

Bioinformatic survey for antibiotic susceptibility genes

Gene annotations of the RSA 493 genome were reviewed to identify presence of gene predicted to have involvement in antibiotic susceptibility and cross-referenced with proteins known in *E. coli* to have involvement conferring protection or susceptibility to antibiotics (Table 3-1). Then protein sequences of those genes were then searched in the standard databases of NCBI with NCBI DELTA-BLAST to identify similar proteins encoded by genes in the other nine *C. burnetii* strains surveyed in this study. All hits with greater than 90% amino acid identity were compiled in Table 3-2 linking which strains possessed genes potentially encoding proteins involved in antibiotic

susceptibility for that class of proteins. Strain specific gene locus tags and protein tags are included in the table.

Determining the effects of pH on antibiotic efficacy

Trials were conducted with 1X ACCM-D with the initial pH of 4.75 and 1X ACCM-D with the initial pH of 7.0, pH to compare the differences in antibiotic efficacy during aerobic growth of *E. coli*. Overnight cultures of *E. coli* were growth in either 1X ACCM-D pH 4.75 or 1X ACCM-D pH 7.0, and diluted 1:100 and grown until same OD_{600} is reached (equivalent to $1x10^8$ cells/mL) prior to inoculating $5x10^6$ cells/well in 96-well flat-bottom culture plates with 200 μL/well 1X ACCM-D pH 4.75 or 1X ACCM-D pH 7.0 and bacterial growth was monitored OD_{600} in a plate reader heated to 37°C with shaking of the plate every 15 seconds and absorbance read every minute. Wells were untreated with antibiotics as a control, medium pre-treated one day prior to inoculation, medium pre-treated two days prior to inoculation, or medium treated at the time of inoculation to assess how the pH of the medium affected inhibitory effects of dilution series of ampicillin (Gold Biotechnologies, USA), and chloramphenicol (Gold Biotechnologies, USA), or tetracycline (Millapore Sigma, USA), antibiotic stocks were generated based on concentration of the particular lots of the antibiotics according to the CLSI guidelines (CLSI, 2012)

Determination of bacterial numbers using genome copy numbers and CFUs

Testing of the strains against the antibiotics *in vitro* using the liquid and solidified 1X and 2X ACCM-D (Appendix) mixed 50/50 with 0.5% (wt/vol) agarose, respectively, was

performed to determine inhibitory effects of the antibiotics corresponding to the predicted function of the genes identified in the bioinformatic screen. Trials with avirulent NMII was performed in 96-well plates, stocks were diluted to 5.5 x 10^4 GE/mL in 1X ACCM-2 pH 4.75 with 0.1 mL used as inoculum for each well containing 0.1 mL of 1X ACCM-2 pH 4.75 that was untreated, treated with a dilution series of ampicillin or streptomycin (25 μg/mL, 50 μg/mL, 100 μg/mL). To minimize risk of evaporation of culture media, samples were surrounded by wells of sterile water. The 96-well plate containing the cultures were incubated at 37°C in an atmosphere of 5% CO_2/2.5% O_2 for 4 days. On day four, cultures were diluted 10-fold in 1X ACCM-2 and 5 μL of each dilution was spotted on tilted solidified agarose 1X ACCM-2 plates to allow the spots to elongate into columns and incubated at 37°C 5% CO_2, 2.5% O_2 for 14 days and colonies were counted to determine CFU/mL. Cultures were harvested at day four to assess bacterial numbers by quantitative PCR of the genomic copy numbers of *com1* using published methods (Brennan and Samuel, 2003). Cultures were serially diluted to plate onto solidified agarose plates of 1X ACCM-D to establish viable bacterial numbers to determine the inhibitory effects of aminoglycosides (streptomycin, Millipore Sigma, USA) and β-lactams (ampicillin, Gold Biotechnologies, USA) in accordance with the guidelines established by the CLSI (CLSI, 2012).

Studies on virulent strains were performed by diluting stocks of Scurry Q212, Henzerling RSA331, reference acute isolate, RSA 493 NMI, and the reference chronic isolate, Ko-Q229 were diluted in 1X ACCM-D pH 4.75 to 5x10^6 GE/well with 0.1 mL

aliquoted per well of 96-well plates. Antibiotic dilutions were prepared at twice the plating concentration and 0.1 mL was added to the 0.1 mL of bacterial cultures to reach the final plating concentration and incubated 2 days at 37°C 5% CO_2, 2.5% O_2. Cultures were harvested at day four to assess bacterial numbers by quantitative PCR of the genomic copy numbers of com1 and be serially diluted to plate onto solid agarose plates of 1X ACCM-D to establish viable bacterial numbers to determine the inhibitory effects of aminoglycosides (streptomycin, Millapore Sigma, USA), β-lactams (ampicillin, Gold Biotechnologies, USA), chloramphenicols (chloramphenicol, Gold Biotechnologies), and tetracyclines (tetracycline, Millipore Sigma, USA) in accordance with the guidelines established by the CLSI (CLSI, 2012).

Acknowledgments

The authors would like to thank Dr. Heidari B. Manijeh for the generous donation of the E. coli strain used in this study. Special thanks go to Dr. Travis McCarthy for sharing his time and assistance with facility support in the BSL-3. NPO was supported by the University of Missouri Gus T. Ridgel Fellowship and Life Sciences Fellowship, NIH NIGMS IMSD training grant, and NIH T32 training grant in addition to funds from the College of Veterinary Medicine's Veterinary Pathobiology department.

Chapter 4

Discussion

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Discussion

For a bacterial pathogen that was discovered over eighty years ago, surprisingly little is still known about how this bacterium causes disease in animal hosts. However, since the advent of the axenic ACCM growth media formulations (Omsland *et al.*, 2009; Omsland *et al.* 2011, Sandoz *et al.*, 2016) and genetic tools to manipulate its genome (Beare *et al.* 2011; Beare *et al.* 2014; Sandoz *et al.*, 2016; Beare *et al.* 2018), the *Coxiella* field of researchers has finally begun to lift the veil and continue to develop a better grasp of the bacterial factors and host factors involved in Q fever pathogenesis

The work presented here on the *C. burnetii* protein, Com1, answers key questions about the function of this protein and potential role in Q fever pathogenesis that initially raised when the gene encoding Com1 was first cloned and sequenced (Hendrix *et al.*, 1993). The confirmation that Com1 has biochemical and functional characteristics of protein disulfide isomerases, like DsbC, are the first step into better understanding the molecular biology of an obligate intracellular pathogen that has largely been studied from the perspective of the host immune response to infection by *C. burnetii*. Studies continue to elaborate on the host-pathogen interactions with the use of the new genetic tools by deleting genes that encode *C. burnetii* effector proteins, which is a critical piece in understanding the molecular mechanisms of Q Fever pathogenesis. However, studies that seek to elucidate basic molecular biological processes of *C. burnetii* are rare.

There is considerable value to developing a solid understanding of the biological

processes of pathogen, since that knowledge directly can translate to devising creating new approaches to therapeutic alternatives which are sorely needed for *C. burnetii*. Continuing and the research into *C. burnetii* Dsb proteins will provide a comprehensive picture of the importance of such proteins in basic biological functions for the bacterium and the role of the proteins in essential virulence factors with multiple cysteine residues that require those Dsb proteins to carry out virulence functions. From a broad view, this growing field of research on the role of Dsb protein in virulence offers an exciting opportunity for the the development of novel therapeutic targets against Dsb proteins. Research has already begun in this direction with the development of small molecule inhibitors that have the potential for being effective against a wide array of bacteria since the active sites of Dsb protein active site are so highly conserved (McMahon *et al.*, 2014; Adams *et al.*, 2015; Duprez *et al.*, 2015; Halili *et al.*, 2015; Landeta *et al.*, 2019).

So very little is know as to how *C. burnetii* is able to persist in a host despite intensive antibiotic treatment. This arena contains enormous growth potential for making invaluable contributions to the Q Fever field. Through the application of transcriptomic and proteomic studies, a well designed study would identify what proteins and bacterial regulatory processes are needed to respond to antibiotic exposure. This offers the potential for the use of inhibitors to block the mechanisms *C. burnetii* employs to persist in hosts treated with antibiotics. Most importantly, these studies will reveal alleles that are responsible for conferring resistance or susceptibility to antimicrobials and provide much needed diagnostic targets for clinical laboratories to utilize in order

to determine the antibiotic susceptibility of isolates from patients without the need to culture the pathogen. Given that public health practices are currently moving in this direction to incorporate next generation technologies to identify monitor the worsening antibiotic resistance crisis faced worldwide.

It is rewarding to contribute to the ever growing Q fever field by demonstrating that Com1 is a functional protein disulfide isomerase thirty years since the submission of article in which Hendrix *et al.*, first clone and sequence the *com1* gene and raise the important question of what role it has in Q fever pathogenesis. Further studies arising from this work will explore how the Com1 P219T substrate capture mutant effects *C. burnetii* NMII infection of macrophages and mice. These studies will ultimately advance to the stage of determining the consequences of a *C. burnetii com1* deletion phenotype to truly answer how this protein affects the pathogenesis of Q fever.

APPENDIX

Identification of Com1 substrates by Mass Spectrometry

At the time of submission of this dissertation, results from the proteomic analysis of two replicates of the WT Com1 control were obtained and results from the proteomic analysis of two replicates of the P219T Com1 substrate capture mutant were obtained. However, the proteomic analysis from second replicate of the P219T Com1 indicated there was an unexpected loss of protein as the protein samples were processed to remove salt from the buffer; for this reason, another 4 L culture was prepared to isolate proteins for proteomic analysis that are expected following the submission of this dissertation. The last set of replicates for both WT Com1 and P219T Com1 are required for statistical analysis of the protein and those samples are also expected to be analyzed following the submission of this dissertation. A table has been included of the combined results from the two replicates of the WT Com1 proteomic analysis (Table A-1).

Table A-1. List of proteins identified from two WT Com1 control replicates.

Protein Description	Locus Tag	Accession	Identified in P219T Analysis	Cysteines	Hit Frequency
CsrA1 translational regulator	CBU_0024	Q83FB6	No	0	15
Uncharacterized protein	CBU_0065	Q83F83	No	1	9
Uncharacterized protein	CBU_0089a	B5QS73	Yes	2	6
CpoB cell division coordinator	CBU_0092	Q83F57	No	1	57
Uncharacterized exported protein	CBU_0110	Q83F41	Yes	7	8
UPF0234 protein	CBU_0114	Q83F37	No	0	5
NusG transcription termination protein	CBU_0225	Q83ET5	No	0	3
50S ribosomal protein L7/L12	CBU_0229	P0C8S3	Yes	0	18
Tuf-2 elongation factor Tu	CBU_0236	Q83ES6	Yes	2	5
50S ribosomal protein L23	CBU_0240	Q83ES2	No	1	8
30S ribosomal protein S19	CBU_0242	Q83ES0	No	0	4
50S ribosomal protein L24	CBU_0249	Q83ER5	No	0	3
30S ribosomal protein S13	CBU_0260	P59753	Yes	1	13
DNA-directed RNA polymerase subunit omega	CBU_0302	Q83EL6	No	1	21
Aminopeptidase N	CBU_0338	Q83EI2	No	12	2
Hypothetical cytosolic protein	CBU_0340	Q83EI0	No	2	3
50S ribosomal protein L27	CBU_0386	Q83ED9	No	0	5
30S ribosomal protein S20	CBU_0389	Q83ED6	No	0	5
Aspartate 1-decarboxylase	CBU_0422	Q83EA4	No	0	3
30S ribosomal protein S16	CBU_0445	Q83E83	Yes	1	15
Exodeoxyribonuclease 7 small subunit	CBU_0468	Q83E62	No	1	11
DUF2007 domain-containing protein	CBU_0469	Q83E62	No	1	17
Phospholipase A1	CBU_0489	Q83E43	No	2	2
50S ribosomal protein L32	CBU_0491	Q83E41	Yes	0	29
Acyl carrier protein	CBU_0496	Q83E38	No	0	7
Uncharacterized protein	CBU_0510	Q83E24	No	0	28
Uncharacterized protein	CBU_0516a	B5QS96	Yes	7	4
ComE competence operon protein 1	CBU_0532	Q83E05	Yes	0	10
Uncharacterized protein	CBU_0562a	B5QS99	Yes	7	93
Ferredoxin	CBU_0581	Q83DW1	No	9	7
OmpH outer membrane protein	**CBU_0612**	**Q83DT1**	**Yes**	**1**	**979**
FKBP-type peptidyl-prolyl cis-trans isomerase	CBU_0630	P51752	Yes	0	75
Uncharacterized protein	**CBU_0632**	**Q83DR4**	**Yes**	**1**	**106**
6 7-dimethyl-8-ribityllumazine synthase	CBU_0648	Q83DP8	No	3	6
Nucleoid-associated protein	CBU_0656	Q83DP1	No	0	4
Uncharacterized exported protein	CBU_0731	Q83DJ9	Yes	6	7
Ribosome-associated factor Y	CBU_0745	Q83DI6	No	1	5
Periplasmic serine endoprotease DegP-like	CBU_0755	Q83DH6	Yes	0	4
Uncharacterized protein	CBU_0802	Q83DD6	Yes	0	6
50S ribosomal protein L9	CBU_0867	Q83D73	No	0	2
Enhanced entry protein	CBU_0915	Q83D29	Yes	5	47

Protein Description	Locus Tag	Accession	Identified in P219T Analysis	Cysteines	Hit Frequency
UPF0422 protein	CBU_0937	Q83D09	Yes	5	7
Bcp, putative peroxiredoxin	CBU_0963	Q83CY8	No	4	40
CsrA-2 translational regulator	CBU_1050	Q83CL9	No	0	24
Uncharacterized exported protein	CBU_1095	Q83CL9	Yes	5	10
Alpha-acetolactate decarboxylase	CBU_1097	Q83CL7	Yes	0	9
Uncharacterized protein	CBU_1173	Q83CE6	Yes	4	45
Glycine-rich RNA-binding protein	CBU_1183	Q83CD7	Yes	0	64
Translation initiation factor IF-1	CBU_1195	Q83CD1	No	0	28
Chaperone protein DnaK	CBU_1290	O87712	Yes	2	10
Integration host factor subunit alpha	CBU_1320	Q83C16	No	0	4
50S ribosomal protein L35	CBU_1324	Q83C12	Yes	0	8
Translation initiation factor IF-3	CBU_1325	Q83C11	No	1	12
Uncharacterized exported protein	**CBU_1366**	**Q83BX1**	**Yes**	**1**	**118**
Ribosome-recycling factor	CBU_1383	Q83BV4	No	1	11
Dihydrolipoyllysine-residue succinyltransferase	CBU_1398	Q83BU7	No	1	6
Uncharacterized exported protein	CBU_1404	Q83BU6	Yes	7	42
17 kDa common-antigen	CBU_1425	Q83BS7	Yes	3	3
Uncharacterized protein	CBU_1429a	B5QSD3	Yes	8	10
HupB DNA-binding protein HU	**CBU_1464**	**Q83BN9**	**Yes**	**0**	**66**
Asn/Gln-tRNA amidotransferase subunit C	CBU_1473	Q83BN0	No	0	3
SecB protein-export protein	CBU_1519	Q83BI9	No	1	12
Glutaredoxin	CBU_1520	Q83BI8	No	2	28
Carboxy-terminal processing protease	CBU_1538	Q83BH0	Yes	0	4
CxxC_CXXC_SSSS domain-containing protein	CBU_1558	Q83BF2	No	4	13
Hypothetical membrane spanning protein	CBU_1576	Q83BD5	No	6	5
30S ribosomal protein S21	CBU_1593	Q83BB9	Yes	1	2
DotH (IcmK) T4BSS component	CBU_1628	Q83B85	Yes	1	7
Uncharacterized protein	CBU_1634a	B5QSE4	No	0	3
DotA, T4BSS component	CBU_1648	Q83B67	Yes	7	3
Hypothetical membrane associated protein	CBU_1651	Q83B64	No	5	3
IcmX, T4BSS component	CBU_1652	Q83B63	Yes	0	13
Hypothetical cytosolic protein	CBU_1677	Q83B41	No	1	9
Uncharacterized protein	CBU_1705	Q83B15	Yes	0	4
Superoxide dismutase [Fe]	CBU_1708	P19685	No	2	10
Glycine cleavage system H protein	CBU_1715	Q83B07	No	0	12
Chaperonin GroEL	CBU_1718	P19421	Yes	2	2
Co-chaperonin GroES	**CBU_1719**	**P19422**	**Yes**	**0**	**315**
Uncharacterized protein	CBU_1764a	B5QSF7	No	6	4
Glyceraldehyde-3-phosphate dehydrogenase	CBU_1783	Q83AU5	Yes	4	4
Superoxide dismutase [Cu-Zn] SodC	CBU_1822	Q83AQ8	Yes	2	45
50S ribosomal protein L25	CBU_1840	Q83AP1	No	2	2
Uncharacterized exported protein	CBU_1847	Q83AN5	Yes	0	55
Uncharacterized protein	CBU_1847b	B5QSG2	Yes	6	44

Protein Description	Locus Tag	Accession	Identified in P219T Analysis	Cysteines	Hit Frequency
Non-proteolytic protein, peptidase family M16	CBU_1901	Q83AI5	Yes	0	4
Com1 *Coxiella* outer membrane protein 1	**CBU_1910**	**H7C7D7**	**Yes**	**2**	**94**
Uncharacterized protein	CBU_1930a	B5QSG7	No	9	17
RNA polymerase-binding transcription factor DksA	CBU_1969	Q83AD5	No	4	10
Uncharacterized exported protein	**CBU_1984**	**Q83AC0**	**Yes**	**8**	**283**
Uncharacterized exported protein	CBU_2072	Q83A39	Yes	4	14
Thioredoxin	**CBU_2087**	**Q83A24**	**Yes**	**2**	**123**

Table A-1. List of Proteins Identified from WT Com1 Control. List comprises the duplicate hits found between the two analyses of the WT Com1 control replicates. A total of 94 protein hits were identified from both analyses. Results highlighted bold indicate the hits are among the top ten hit frequencies in both WT Com1 control replicates.

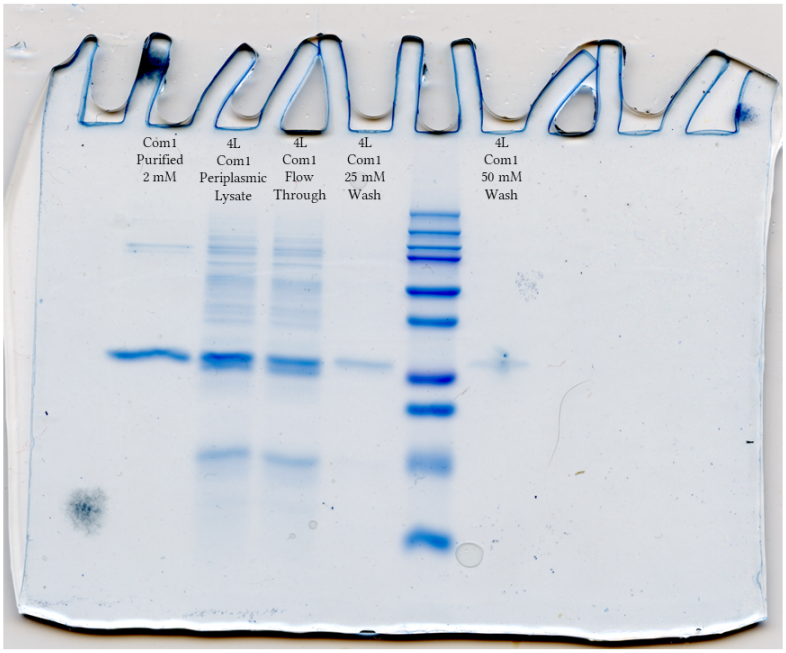

Figure A-1. Typical elution of periplasmic isolates from 4 L Com1 culture. A 15% SDS-PAGE analysis of purified Com1 control and 25 μL of periplasmic protein isolates mixed with 2X Laemmli sample buffer (Bio-Rad) and run at 150 volts for 1 hour and visualized with GelCode Blue Safe Protein Stain (Thermo Scientific).

Localization of Com1 using transmission electron microscopy

At the time of submission of this dissertation, numerous attempts have been made to establish the localization of tagged recombinant Com1 protein in *C. burnetii*. Conditions had been established by the MU Electron Microscopy Core to embed fixed cultures of *C. burnetii* in resin (LR White). However, repeated trials using different primary and secondary antibody dilutions to stain the sections for localization studies were inconclusive. In an effort to troubleshoot the issues, *E. coli* strains carrying plasmids that express the tagged recombinant Com1 were induced as described in the methods in Chapter 2 and fixed as described in the localization methods and submitted to the MU Electron Microscopy Core to embed in resin and create sections for additional staining trials. The results from this control are expected to be obtained after the submission of this dissertation and should provide some insight into the inconclusive results obtained from the attempts to determine localization of Com1 in *C. burnetii*. Based on the bioinformatic analysis of the Com1 protein sequence, a predicted signal sequence was identified that suggest Com1 is trafficked to the bacterial membrane. A tagged recombinant Com1 with the tag preceding the signal sequence is expected to lose the tag after localization to the membrane region when the tagged signal sequence is cleaved. A tagged recombinant Com1 with the after the signal sequence cleavage site is expected to retain the tag after localization to the membrane region. Western blot analysis of the periplasmic protein isolation from *E. coli* strains containing plasmids either of the two tags revealed only the Com1 27 kDa band present when the tag was located after the signal sequence cleavage site (Figure A-1). The confirmation that tagged Com1 and not the tagged signal sequence are found after periplasmic isolation

of proteins demonstrates that Com1 localizes to the membrane region of the bacteria. Additional work is needed to clarify where in the membrane region Com1 localizes.

Table A-2. Acidified citrate cysteine medium-defined (ACCM-D) formulation pH adjusted to 4.75 and passed through 0.22 micron filter unit.

	Concentration (mM)
L-Alanine	1.26
L-Arginine monohydrochloride	0.75
L-Asparagine	0.67
L-Aspartic acid	0.54
L-Cysteine hydrochloride monohydrate	1.56
L-Glutamine	2.44
L-Glutamic acid potassium salt monohydrate	3.31
Glycine	1.17
L-Histidine	0.35
L-Isoleucine	0.85
L-Leucine	1.76
L-Lysine monohydrochloride	1.43
L-Methionine	0.46
L-Phenylalanine	0.63
L-Proline	3.02
L-Serine	1.68
L-Threonine	1.02
L-Tryptophan	0.25
L-Tyrosine	0.6
L-Valine	1.37
Citric acid	13.4
Sodium citrate	16.1
Potassium phosphate	3.7
Magnesium chloride	1
Sodium chloride	124.7
Calcium chloride	0.09
Iron sulfate	0.01
Methyl-b-cyclodextrin	1 mg/mL
RMPI Powder without amino acids	1 mg/mL

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VITA

Nicholas Paul Olivarez, born in Independence, Missouri, where he spent his early years before moving to Elk Grove Village, Illinois, where he was raised until setting off on his own for over two decades. His awakening began at The Evergreen State College in Olympia, Washington where he fell in love with the Pacific Northwest, realized his passion for molecular and microbiology, and learned the value of independence and knowing how to do anything required in a laboratory, under the tutelage of the imminent bacteriophage biologist, Dr. Betty Kutter.

After pursuing his Master's of Science degree at the Virginia Commonwealth University in Dr. Gail E. Christie's phage lab, he sought to progress to working with viruses that infect humans. While working as a Research Specialist at the University of North Carolina – Chapel Hill in Dr. Aravinda de Silva's Dengue virus research lab, he made significant contributions to the field with his novel methods he developed for antigen production. Returning to the Pacific Northwest, he worked as a Research Scientist and managing the host-pathogen laboratory of Dr. Joshua Woodward, who was grateful enough to appreciate Nicholas's intent to pursue his Ph.D. at the earliest opportunity.

Nicholas accepted the offer from the Molecular Pathogenesis and Therapeutics Ph.D. program at the University of Missouri, where his years of research experience

resulted in him creating numerous independent research projects and pursuing them single-handedly, while making time share new culture methods with his lab mates in Dr. Guoquan Zhang's Q Fever laboratory. His ambitious research projects required his strengths in biochemistry, bioinformatics, virology, and microbiology. Despite encountering a series of technical and global public health crises, his focus allowed him to rebound and execute challenging experimental goals that will lay the foundation for future work in the Q Fever field.

Nicholas has accepted an offer for the 2022 Association of Public Health Laboratories – CDC Infectious Disease Fellowship program where he will begin his career as a public health professional, fusing his passions for science and service to serve those in need.