# *COXIELLA BURNETII:* INTRANASAL IMMUNIZATIONS AND AXENIC MEDIA ADAPTATIONS

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2013

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2014

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 2020

COXIELLA BURNETII INTRANASAL IMMUNIZATIONS AND AXENIC MEDIA ADAPTATIONS

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ACKNOWLEDGEMENTS

I, of course, must start by thanking my family. To Molly and Rhett, you have completely changed my life for the better. While many may say that they can't imagine pursuing a Ph.D. with kids, I can't imagine having done any of this without the two of you. I love you both so much and I hope that you always know you can do anything. To Ryan, none of this would have been possible at all without your love and support. You were my rock on the hard days, and my favorite person to celebrate with on the good ones. I wouldn't be where I am today at all without the help of my parents, Barbara and Jim Peters. You two raised two daughters, and both became doctors in the same summer. Thank you for everything you taught us, and everything you've done for us to help support and get us here. I also couldn't have done this without my sister, Caty. You've been my best friend since the day you were born and I'm so happy we've had each other to celebrate all of life's milestones, big or small. I also have to thank Andrea Lenhart and JaNeel Johnson, who may not be biological family but treat my kids as though they are their own. I truly cannot ever thank you enough for your help in easing the mom guilt and making my kids feel so loved.

There are also many people at OSU without whom this would not have been possible. First of all, to Dr. Ed Shaw, thank you for always being my mentor. Not just in the lab, you've been an excellent mentor in all of the struggle's life had to offer over the past 6 years and I'll forever be thankful for the years I spent in your lab. To Dr. Lutter, thank you for stepping in and treating me like one of your own students in the year after Dr. Shaw left. You helped me to make the best out of what I thought would be a difficult situation and helped me whenever I needed it. I'm proud to be a graduate of both the Shaw and Lutter labs. To the rest of my committee, Dr. Patrauchan, Dr. Prade, Dr. Wozniak, and Dr. Oomens, thank you for all of the support and advice over the years. I would also like to thank Dr. Conway and the rest of the department for all of their kindness and support.

I also have so many friends I've made in my years at OSU that I must thank. To Jorge, thanks for being my venting buddy over countless bowls of queso or fried mushrooms. Grad school wouldn't have been the experience it was without you. Jerreme, thanks for always being willing to help me out with experiment advice, or just opening bottles I couldn't get by myself. Michelle, we actually did it! We came in together and finished together and I'm so thankful I had you to experience all of this alongside me. To Payton, thank you for all of your guidance and friendship that challenged me to become a better teacher.

Finally, thank you to the community of both Stillwater and Perkins. I will forever be thankful for the friendships we made at Center Church as well as Renaissance and Perkins Schools. Thank you for making us feel so at home for the past 6 years, and for all of the love and support.

Name: MELISSA N. BREWER

Date of Degree: JULY, 2020

Title of Study: COXIELLA BURNETII INTRANASAL IMMUNIZATIONS AND AXENIC MEDIA ADAPTATIONS

Major Field: MICROBIOLOGY AND MOLECULAR GENETICS

**Abstract:** *Coxiella burnetii* is an obligate intracellular pathogen and the causative agent of the zoonotic illness Q fever. With an infectious dose of 1-10 organisms, *C. burnetii* is one of the most infectious bacteria known. Formulations of killed whole-cell *C. burnetii* virulent strains has been shown to be effective when administered intramuscularly, intraperitoneally, and sub-cutaneously, but has not been assessed using an intranasal route of inoculation that would also bring to bear the mucosal immune response. We hypothesized that intranasal immunization would be protective against subsequent challenge with live virulent *C. burnetii.* Using a guinea pig model, intranasal immunization with a mixture of killed whole-cell *C. burnetii* Nine Mile phase I combined with double mutant lethal toxin (DmLT) adjuvant. Booster immunizations were administered at 14 and 28 days. Sera was drawn for IgG titers prior to, and at 14, 28, and 56 days following intranasal immunization. Analysis of sera indicates that a robust humoral response is elicited following intranasal immunization. Additionally, immunized guinea pigs that were subsequently challenged with virulent *C. burnetii* NMI did not develop significant clinical symptoms as measured by fever and weight loss when compared to animals receiving the mock immunization. Studies defining minimal immunization requirements, potential hypersensitivity response, and protective antigen profile using this physiologically relevant exposure route will aid us in defining a protective response to this unusual aerosol acquired pathogen.

For the first 80 years following its discovery, *C. burnetii* could only be grown in a system providing live cells within which it would replicate. The relatively recent advent of cell free media has been a boon for *C. burnetii* research on many fronts. We noticed when infecting cultured cells using *C. burnetii* that had been repeatedly passaged in cell free defined acidified citrate cysteine media (ACCM-D), that the infectivity rates were lower than expected based on the number of genomes used to initiate the infection relative to *C. burnetii* that had been derived from infected cells. Infectious assays comparing *C. burnetii* from passages 1, 3, 5 and 10 determined that by passage 10 the organism's relative ability to infect cultured cells had decreased 2-logs. We hypothesized that molecular changes were occurring in these early passages that related to the ability of the bacteria to infect or grow within host cells at their normal rate. We initiated a "Reverse Evolution" approach to study the molecular changes that occur following serial passages in axenic media to determine what occurs when the stressors associated with *C. burnetii's* typically intracellular lifestyle are removed. We found that the concentration of DotA and IcmX, two structural proteins of the Type IVB Secretion System, which is required for intracellular growth, were significantly reduced by passage 10. Mass spec analysis of passages 1, 3, and 5 also demonstrate changes between protein levels in subsequent passages analyzed. Expanded mass spec and RNAseq analysis to include passage 10, along with immunoblot analysis and genome sequencing of both early and longer-term passages will elucidate molecular mechanisms required for *C. burnetii's* intracellular survival and growth.

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# CHAPTER I

## INTRODUCTION: SCOPE OF STUDY

*Coxiella burnetii* is an obligate intracellular bacterium in nature. It is the causative agent of the zoonotic illness Q fever [1]. Acute Q fever is characterized by mild flu-like symptoms including headache, fever, coughing, and myalgia. This illness typically resolves on its own although in diagnosed cases, a 14-day course of doxycycline may be prescribed [2]. Chronic infections may also occur and are accompanied by symptoms that include endocarditis, hepatitis, or chronic fatigue syndrome [3, 4]. Treatment of chronic Q fever is more complicated and expensive, with an 18-24-month course of doxycycline and hydroxychloroquine being a typical treatment [5].

*Coxiella burnetii* undergoes a biphasic lifecycle composed of two morphological forms, the small cell variant (SCV) and the large cell variant (LCV) [6, 7]. The SCV is the environmentally stable form of the organism that infects eukaryotic host cells. Once within the host cell, *C. burnetii* remains within a cell derived vacuole that essentially follows the endocytic pathway to become a pathogen containing vacuole resembling a late endosome. These are referred to as the parasitophorus vacuole (PV). Within this vacuole, *C. burnetii* transforms into the replicative LCV form within 24-48 hours post infection. The LCV replicates within the PV while manipulating the host cell to provide the necessary nutrients, etc. for its survival and replication. After 6 days, depending on initial infection level, *C. burnetii* begins an asynchronous transformation back to the SCV form, and eventual cell lysis frees the infectious particles to repeat the process in new host cells [6, 8].

In this dissertation, I sought to address two distinct questions associated with *C. burnetii* infections and molecular host-pathogen interactions, respectively:

- Does intranasal inoculation using killed whole cell *C. burnetii* protect against subsequent challenge using live virulent *C. burnetii*?
- What temporal gene expression and/or genetic changes may occur when the stressors of an intracellular environment are removed?

The environmental stability, aerosol transmission, and an infectious dose of 1-10 organisms [9] are historically of concern and has led *C. burnetii* to be classified by the Centers for Disease Control as a Class B Select Agent [10]. There is an existing commercial vaccine, Q-Vax, that uses killed whole cell *C. burnetii*, however, it is only available in Australia [2, 11-13]. The Q-Vax vaccine is very effective but causes a delayed type hypersensitivity (DTH) response at the site of injection in individuals with previous exposure to *C. burnetii* [14]. This results in the need to screen all individuals prior to receiving this vaccination [15]. As such, there is an ongoing need for a more suitable *C. burnetii* vaccine that removes the need for pre-screening. Whole cell killed formulations of virulent *C. burnetii* strains have been found to be effective following intramuscular, intraperitoneal, and subcutaneous immunization in various infectious challenge models [2]. However, analysis of an intranasal route of immunization for this aerosol acquired infection has not been tested. As such we sought to answer the following question:

- Does intranasal inoculation using killed whole cell *C. burnetii* protect against subsequent virulent *C. burnetii* challenge?

*C. burnetii* was initially discovered and continually cultivated in guinea pigs during the 1930s [2, 16, 17]. Due to its nature as an obligate intracellular pathogen, it could not be

cultured on typical bacterial growth media, although methods were soon developed/discovered whereby it could be cultivated to large numbers in embryonated chicken eggs [17]. Years later, the development of immortal tissue culture methods further advanced the availability of *C. burnetii* models for growth and physiologic research, but research with the organism was still restricted to the limitations of host-cell infections [18]. However, in 2009 a cell free media for growing *C. burnetii in vitro,* Acidified Citrate Cysteine Media (ACCM), was developed allowing research away from the host cell background to be performed [19]. When grown in eggs, tissue culture, or ACCM, i.e. outside of an animal host, a well-documented change occurs within the bacterial population [20-22]. When passaged in eggs, tissue culture, or ACCM, genetic mutations occur that results in a severely truncated LPS. Virulent, or phase I, *C. burnetii* possess a full-length lipopolysaccharide (LPS). The LPS mutant *C. burnetii* are referred to as phase II organisms/strains [23-25]. As a result of this truncation, the *C. burnetii* are no longer able to evade the immune system in an animal host and are no longer virulent. However, these phase II organisms retain all of their ability to invade, survive, and grow within a host cell, making them an exceptional model of phase I *C. burnetii* in tissue culture infections and subsequent molecular analysis of pathogen-host cell molecular interactions [23, 24].

When performing infections of eukaryotic cells with the model *C. burnetii* Nine Mile phase II (NMII) strain that had been serially passaged in ACCM several times, we observed what appeared to be a reduced number of infectious forming units relative to what was expected based on the number of organisms used for the infection. Preliminary analysis demonstrated a decrease in the number of infected host cells that appeared associated with an

increase in the number of in vitro ACCM-D passages of the bacteria. This led us to pose the following questions:

- What temporal gene expression changes may occur when the stressors of an intracellular environment are removed?

# CHAPTER II

# LITERATURE REVIEW

## History of *Coxiella*

### Discovery

The first diagnosis of an unknown illness known as query or "Q" fever occurred in Australia. This unexplained illness was investigated by the physician Edward Derrick following periodic outbreaks of a febrile illness in abattoir workers. The symptoms of this illness were inconsistent with other known diseases, and the test results of patients suffering from this illness were negative for influenza, typhus, leptospirosis, and other common fever agents. Derrick was able to successfully passage the organism from the blood of infected patients to guinea pigs, but was unable to cultivate or visualize the organism by traditional means [1].

Derrick sent emulsified liver from an infected guinea pig to the prominent virologist MacFarlane Burnet after concluding that the pathogen was a virus. Burnet used the liver emulsion to infect animals and document their symptoms. Smears made from the enlarged spleens of infected mice showed intracellular organisms similar to Rickettsiae [16]. Based on these observations Derrick named the Q fever agent *Rickettsia burneti* [26].

Derrick began to look into the possibility that arthropods or other vectors were involved in *R. burneti* transmission. Concurrent with the studies in Australia, a lab in the United States was unknowingly looking into the same organism. The Rocky Mountain Laboratories (RML) in Montana, a part of the federal Dept. of Health Services, were investigating the epidemiology of Rocky Mountain Spotted Fever (RMSF) causative agents [27]. Along with many other studies, Rocky Mountain wood ticks (*Dermacentor*

*andersoni*) were collected along Nine Mile Creek and allowed to feed on guinea pigs. These guinea pigs would then be monitored for fever as a symptom of any potential illness. Following one of these feedings, one of the guinea pigs developed a febrile illness that was transferable to healthy guinea pigs through blood transfusion. However, the symptoms displayed were not consistent with RMSF [28]. This organism was, for the time, named Nine Mile Agent (NMA) as they continued to study and characterize it. Herald Cox was assigned to assist in the characterization. He found that NMA could be passaged to guinea pigs from an infected guinea pig and could be passed through filters that typically retained bacteria, but not viruses [28].

Cox sought to further understand this organism, which he initially called *Rickettsia diaporica* (Greek for "ability to pass through") after initial tests of NMA fit the broad definitions used to classify Rickettsial species. Rolla Dyer, who was the director of the Public Health Service at the time, travelled to RML to verify research claims that were being made by Cox about *R. diaporica*. During Dr. Dyer's time at RML he was accidentally infected with *R. diaporica* while handling infected guinea pigs and embryonated eggs. He used this opportunity to record details about his symptoms and also infected guinea pigs with the agent using his own blood [29]. Following Dyer's publications, guinea pigs that had recovered from *R. burneti* (the Australian agent) challenge were found to have protective immunity against *R. diaporica*. Thus, it was Dr. Dyers accidental infection and subsequent publication that led to the connection between *R. diaporica* and *R. burneti*, and the conclusion that they were the same organism [26, 30, 31].

An outbreak of Q fever during the course of World War II [32, 33] prompted new interest in *C. burnetii* research [34-36], and led RML researcher Cornelius Philip to suggest a single name for *R. burneti* and *R. diaporica*, as well as a new genus to separate this organism from other Rickettsiae sp. due to its unique characteristics. In honor of both Burnet and Cox the subgenus *Coxiella* was suggested with the species name *burneti*, which was later changed to *burnetii* [37].

**Cultivation History and Development**

The growth and cultivation of *C. burnetii* has changed greatly over the course of 80 years and is summarized in Figure 1**.** When *C. burnetii* was first discovered it was only able to be passaged or maintained using infected animals, primarily guinea pigs [2, 16, 17]. Attempts to cultivate the organism on other growth media were unsuccessful [1]. While this allowed researchers to study the infection and symptoms, it was impractical for long term cultivation and maintenance of *C. burnetii* as guinea pigs would eventually either recover and clear the organism or succumb to infection [16, 38]. While studying this organism at RML, Cox discovered he was able to grow *C. burnetii* in a tissue culture of minced embryonated eggs. This led to the subsequent discovery that embryonated eggs could be directly infected and used for larger scale cultivation of *C. burnetii* [17]. Yolk sacs of these infected eggs could be harvested and *C. burnetii* could be purified using differential and density gradient centrifugation [39]. While this was a far better method than passaging in guinea pigs, given the large quantities of *C. burnetii* produced there were safety concerns associated with yolk sac harvesting, as well as the time constraints associated with this method.

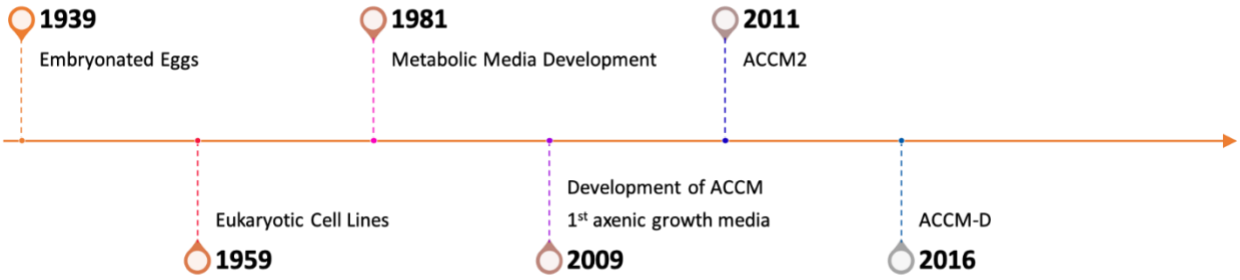


**Figure 1. Timeline of *C. burnetii* Cultivation.** Initial research involving *C. burnetii* was only done in animal models. Advancements in the cultivation of *C. burnetii* over time are demonstrated here (Timeline not drawn to scale).

The use of embryonic tissue culture was an appealing alternative to the previous methods of cultivation [18], and the advent of diverse and robust tissue culture methods has aided in *C burnetii* studies since their development in the late 1950s. Since that time, *Coxiella burnetii* has been shown to infect and grow in multiple mammalian cell lines [18] *in vitro*, as well as at least one tick cell line [40]. Cell culture infection was an advantageous development that allowed for both studies of the organism as well as propagation and isolation of *C. burnetii* using a variety of lysis and differential centrifugation methods. More recently, a methodology of *C. burnetii* isolation from infected cultures was developed where the organisms were driven toward the SCV form prior to bacterial harvest [18], allowing for the initiation of a more synchronous infection in subsequent studies [41]. While these methods showed improvement to previous methods, the requirement of separating *C. burnetii* from residual host cell debris remained a challenge for many molecular studies.

Over the years, researchers have sought the development of a host-cell free means to grow *C. burnetii.* One advancement toward this goal was the development of a media that allowed *in vitro* metabolic activity of purified *C. burnetii*. In this early metabolic media, a relatively minimal metabolic activity could be achieved relative to that observed in host cells [42]. However, advances in, and use of, this metabolic media revealed several of the lysosomal characteristics and physiologic properties of the parasitophorus vacuole (PV) where *C. burnetii* resides during infection [43, 44]. Based on these findings it was demonstrated that *C. burnetii* metabolism improved in conditions similar to those found within the PV. Macro-molecular synthesis of protein and nucleic acid was achieved in simple phosphate buffers when the pH was lowered to 4.5 and supplemented with amino acids and glucose [45, 46]. Proteins were synthesized [46] and secreted [47] for 24 hours post inoculation and DNA synthesis occurred for up to 15 hours [45] in this media. However, cell division did not occur.

These early metabolic media formulations were phosphate based, but in 2008 an improved metabolic media, designated complex *Coxiella* media (CCM), was developed that was citrate based in an effort to appropriately buffer the acidic pH required by *C. burnetii* during extended incubation [48]. The CCM media increased the metabolic activity of purified *C. burnetii* substantially, yet bacterial replication was not detected [19]. Further studies and bioinformatic analysis of the *C. burnetii* genome revealed that *C. burnetii* is auxotrophic for several amino acids that were subsequently added to create Acidified Citrate Cysteine Medium (ACCM) [19, 48]. An increase in protein synthesis resulted, but *C. burnetii* replication remained elusive. Further gene expression and bioinformatic analysis of global *C. burnetii* mRNA expression during ACCM metabolism

vs. cell culture growth suggested that *C. burnetii* possesses a cytochrome system similar to that of microaerophilic bacteria [19]. When *C. burnetii* was subsequently incubated in ACCM in microaerophilic conditions (2.5% oxygen), replication was achieved [19]. The growth in ACCM was shown to generally mimic the biphasic life cycle of *C. burnetii*, with the change from SCV to LCV followed by the conversion back to SCV after log growth, as observed in tissue culture infections [19]. This original ACCM contained complex ingredients such as RPMI media, yeast extract, and fetal bovine serum (FBS), potentially complicating downstream molecular analysis [19]. However, in 2011, a modification of the components in ACCM led to an updated media, ACCM-2, that did not contain Fetal Bovine Serum (FBS) [49]. The replication of *C. burnetii* in ACCM-2 increased between 1 to 2 logs versus ACCM while the generation time was slightly decreased. In addition, with the development of ACCM-2 came the ability to prepare agar plates that could be utilized for the growth of *C. burnetii* colonies [49]. Bacterial numbers and generation times were further improved with the most recently developed media, ACCM-D [50]. ACCM-D is a fully nutritionally defined media with known amino acid concentrations [50, 51]. This defined media also allows researchers to take advantage of *C. burnetii's* auxotrophy to generate plasmid systems that enable selection using non-antibiotic methods to create stable genetic mutants [52]. Given the paucity of selectable antibiotic markers allowed in *C. burnetii* genetics this was a particularly important development.

## Q fever

Q fever is a zoonotic illness caused by the intracellular bacterium *C. burnetii*. It is found worldwide in the environment and has the ability to infect a broad range of organisms [2, 53]. Transmission of *C. burnetii* generally occurs when contaminated aerosols are inhaled following release from an infected organism. In humans these inhaled *C .burnetii* infect alveolar macrophages and initiate systemic infection as monocytes migrate from the lungs to other sites [8, 54]. *C. burnetii* is environmentally stable and has an infectious dose of only 1-10 organisms being required to initiate an infection [55]. The symptoms of *C. burnetii* vary greatly, but typically present as a mild flu-like illness. Severity of illness and presentation can vary from individuals who may be asymptomatic to those developing chronic infections where major organs, including the heart and liver, can be affected [2, 12].

### Acute illness

The incubation period of Q fever ranges from 1 to 3 weeks and varies based on the dose of inoculum. Many Q fever cases are asymptomatic, and symptomatic illness most commonly presents as a self-limiting flu-like illness [2, 56, 57]. Patients typically experience an abrupt onset of fever, fatigue, chills, and headaches. The fever can reach 104-105 F and peaks within 2-4 days before returning to normal between 5-14 days. Patients older than 40 years have been reported to have a longer duration of fever [2, 58]. When Q fever is left untreated the fever has been reported to last from anywhere between 5 to 57 days. One quarter of Q fever patients experience a biphasic fever, where

following the first phase there is a reappearance of the fever lasting from 1 to 19 days [2, 56, 57, 59].

Pneumonia is a common complication associated with acute Q fever. Patients experiencing high fever frequently develop atypical pneumonia. This is often associated with fatigue, chills, myalgia, and sweats [60]. Hepatitis, myocarditis, pericarditis, and meningoencephalitis occur less frequently than pneumonia but have also been reported [2]. Acute or asymptomatic Q fever can also cause pregnancy complications that result in spontaneous abortions or placental abruptions [61-63]. The fatality rate associated with acute Q fever is less than one percent, indicating that the normal immune response is capable of eliminating the organism[64].

Diagnosis of acute Q fever is complicated by its flu-like symptoms, which closely resemble other infectious diseases, often leading to misdiagnosis [30, 56, 65-67]. In the majority of acute cases of Q fever, the leukocyte count remains in the normal range [6]. Because of this, serology is the primary method of confirmatory diagnosis. Indirect Fluorescent Antibody assay microscopy is typically used to detect antibodies against both phase II and phase I antigens [68]. An increased titer against phase II antigen is more indicative of an acute infection, while the presence of increased titers against both phase I and phase II antigens can indicate the occurrence of chronic illness [30, 56, 57, 65-68]. Previous treatment of acute Q fever used antibiotics such as tetracycline, cotrimoxazole, ofloxacin, and pefloxacin, or doxycycline for 14-21 days [2, 11, 69, 70]. Combination therapy with doxycycline and chloroquine or hydroxychloroquine for 14-21days is an effective alternative to using antibiotics alone [2, 5, 11, 12, 70].

**Chronic Illness**

Approximately five percent of *C. burnetii* infections manifest as chronic Q fever. The majority of patients developing chronic illness are over the age of 40. Chronic illness can develop anywhere from a month to several years following exposure. Individuals who develop chronic Q fever may have previously experienced acute Q fever or may have been asymptomatic following initial exposure [2, 71-75]. The most common clinical manifestation of chronic Q fever is valvular endocarditis. As many as 60-70% of chronic Q fever cases are characterized by endocarditis and the condition can be fatal if left untreated. Patients with underlying heart disease or previous cardiac valve defects account for 90% of endocarditis cases suggesting that damaged tissue provides a point of adhesion for *C. burnetii* infected cells. Chronic *C. burnetii* endocarditis typically affects aortic or mitral valves, although cases of Q fever endocarditis on prosthetic valves have also been reported [2, 56, 73-76]. Clinical symptoms of chronic Q fever endocarditis include low grade fever, cardiac valve dysfunction, fatigue, weakness, chills, anorexia, weight loss, and night sweats. Cardiomegaly, ventricular hypertrophy, and arrhythmia can be detected via chest x-rays and electrocardiography. Patients with Q fever endocarditis can have peripheral manifestations such as purpuric rash in mucosa and extremities, as well as digital clubbing [2, 56, 69, 71, 73, 75, 77-79]. Chronic hepatitis is often reported in association with Q fever endocarditis [2, 80]. If the illness goes untreated for long periods of time patients also suffer from splenomegaly and hepatomegaly. Some cases have also noted mild hematuria that may lead to renal complications [2].

While endocarditis is the most common presentation of chronic Q fever, there are other prominent presentations. These include osteoarticular infections, pulmonary infections, chronic hepatitis, and chronic fatigue syndrome [2]. Although rare, chronic Q fever can present as vascular infections, which can be fatal [2]. Because *C. burnetii* colonizes the uterus, placenta, and mammary glands of infected pregnant women, Q fever has also been associated with intrauterine growth retardation, low birth weight, premature delivery, spontaneous abortion, and stillbirth [2, 65, 69].

Treatment of chronic Q fever is typically doxycycline and hydroxychloroquine for 18 to 36 months. Because of the use of hydroxychloroquine, patients require regular eye examinations to monitor for light sensitivity development [2, 5, 11, 12, 70]. Pregnant women are not treated with doxycycline and chloroquine due to the risk of complications and are instead treated with cotrimoxazole [69].

**Vaccine History**

*C. burnetii* is environmentally stable, typically acquired through an aerosol route, has an infectious dose of 1-10 organisms, and has been weaponized in the past [55]. These factors drove *C. burnetii* to be classified as a Class B Select agent by the Centers for Disease control [10]. This classification makes preventative measures against *C. burnetii* desirable. Preventative measures would include vaccination of both humans and animals, since ruminant livestock are a common reservoir of the organism [2, 11]. Experimental vaccines prepared from both virulent (phase I) and avirulent (phase II) organisms over several decades [81-84] have demonstrated that formalin-inactivated phase I *C. burnetii* is 100-300 times more protective with greater fever reduction and

higher antibody titers than phase II *C. burnetii* in vaccinated guinea pigs [85]. Vaccination of cattle provided protection against *C. burnetii* induced abortion, low fetal birth weight, and infertility, but did not eradicate *C. burnetii* in cattle that had been previously infected with the organism [2, 11]. A vaccine created in Europe that contained a mixture of *C. burnetii* and *Chlamydia psittaci* was also found to be effective against fertility issues in cattle and goats [2, 83, 86], however, humans in contact with these goats still reported developing *C. burnetii* infections. The results of animal vaccinations have been inconsistent, so animals are not currently being routinely vaccinated [2, 86].

For Q fever prevention in humans, there are three different types of vaccines currently available. The first is a Q fever vaccine called Q-Vax, and is prepared from formalin-inactivated Phase I *C. burnetii* Henzerling strain, that is currently only available in Australia [2, 11-13]. This vaccine has been found to be highly protective but causes a delayed-type hypersensitivity (DTH) response when inoculated into individuals with prior *C. burnetii* exposure [14]. Early vaccines given to laboratory workers in the 1940s and 1950s also resulted in a DTH response [13]. Due to this reaction, all individuals must receive a skin test for reactivity prior to receiving the vaccination. The second, in the US, is an Investigational New Drug (IND) that is also phase I formalin-inactivated *C. burnetii* Henzerling strain but is not commercially available. The third is a soluble lipopolysaccharide (LPS)-protein complex chemovaccine that is extracted form phase I cells using trichloroacetic acid. This formulation has been used in Slovakia [2, 11-13, 87].

A chloroform-methanol pretreatment of phase I *C. burnetii* has been shown to reduce the DTH response seen in whole-cell formalin-inactivated *C. burnetii* vaccines, but it also reduces the level of protection [2, 88]. Individuals who are livestock handlers, animal product processors, veterinarians, or work in laboratories with phase I *C. burnetii* are highly recommended to be vaccinated [2, 11, 89, 90]. Research to find a safe and effective Q fever vaccine that does not require skin testing prior to administration remains an ongoing effort.

## Bacterial Life Cycle

### Biphasic Life Cycle

*C. burnetii* is an obligate intracellular organism that is a Gram-negative pleomorphic coccobacillus. It has a biphasic life cycle with two distinct morphological forms, the small cell variant (SCV) and large cell variant (LCV) [6, 91]. The life cycle is summarized in Figure 2. The SCV is environmentally stable and metabolically inactive, measuring 0.2 to 0.5 um in length [6, 7, 91]. It has a thickened peptidoglycan layer, which has been measured to be from 13 to 21 nm in thickness. The SCV can persist in the environment for long periods of time and is resistant to several chemical and physical treatments. Analysis of peptidoglycan content indicates the peptidoglycan protein complex increases by approximately 30% as the organism transitions from its LCV to SCV forms [7, 8, 92]. This contributes to the environmental stability and makes SCVs less sensitive to desiccation, osmotic shock, ultraviolet (UV) light, sonication, and elevated temperatures that would inactivate many other bacteria [6-8].

In nature, the SCV that is thought to typically initiate infection following the inhalation of bacteria containing particles. After uptake of an SCV by an alveolar macrophage in the lung, the bacterium enters the host cells endocytic pathway, and ends up in a modified late endosome, with both early and late endosome markers, termed the parasitophorus vacuole (PV) [93]. Within the PV *C. burnetii* transitions from SCV to LCV, though the exact combination of signals or proteins that initiate this change are unknown [94]. This transition from SCV to LCV occurs during a growth lag phase of approximately 24-48 hours [95] after which the LCV enters logarithmic growth. The LCV has a thinner peptidoglycan layer than the SCV form as approximately 6.5 to 8 nm spans the peptidoglycan as well as the membranes and periplasmic space. The cell wall of an LCV is that of a typical Gram-negative cell, though they stain Gram variable [6, 7, 91]. The transition from SCV to LCV initiates the exponential growth phase where the LCVs replicate at a doubling time of approximately 12.4 hours during growth within the host cell [95]. At approximately 6 days post infection, the bacteria enter a stationary phase begins, and LCVs begin an asynchronous transition to SCVs before host cell lysis [95].

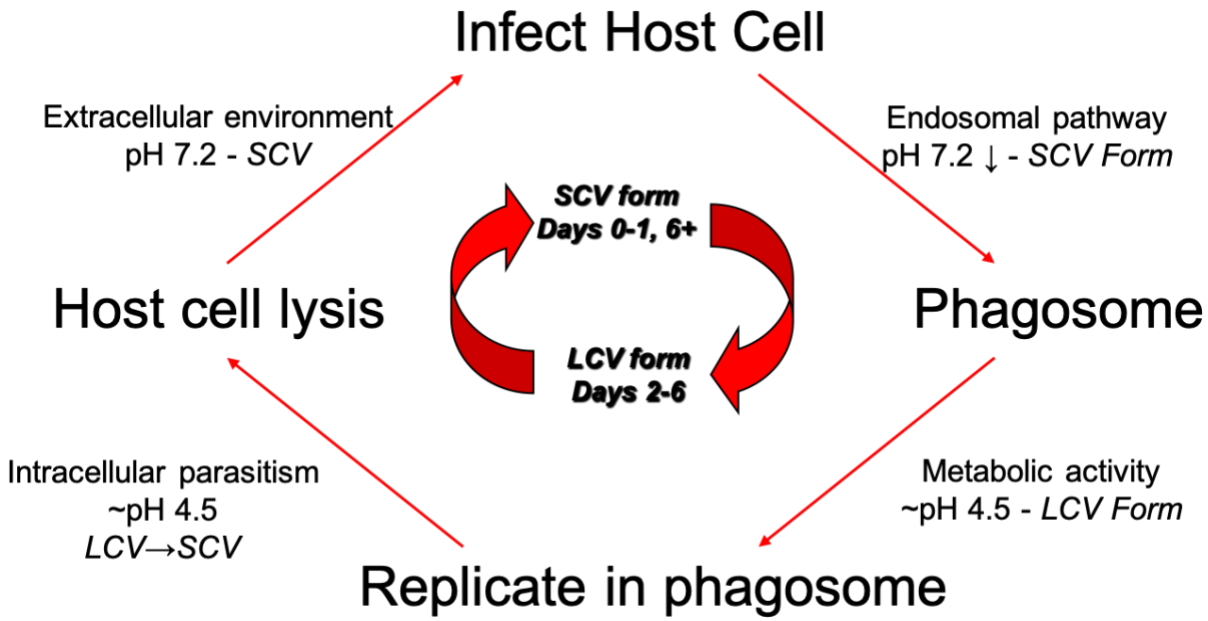


**Figure 2. Intracellular Life Cycle of *C. burnetii*.** The environmentally stable SCV infects a host cell and enters a parasitophorus vacuole via the endosomal pathway where it transitions to the replicative LCV. Prior to host cell lysis *C. burnetii* transitions back to the SCV form.

**Phase Variation**

In nature, *C. burnetii* is an obligate intracellular organism. When isolated from animals in nature or in the laboratory it is virulent and noted for causing disease [8, 42]. When virulent *C. burnetii* is grown in a laboratory setting in embryonated eggs, tissue culture, or ACCM for an extended period of time, bacteria within the population undergo a phase change that results in individual bacteria that are avirulent. The virulent

organisms are referred to as “phase I” and after undergoing the phase change this avirulent form is referred to as “phase II” [8, 42]. The primary phenotypic difference between phase I and phase II *C. burnetii* isolates is lipopolysaccharide (LPS) length. The phase I, or virulent, *C. burnetii* produce a full-length LPS. Phase II *C. burnetii* possess a severely truncated LPS [8, 42]. When animals are inoculated with clonal isolates of phase II bacteria, they are injected into animals it does not cause disease [21].

The best characterized mutation that led to this attenuated virulence is caused by a deletion of 26 kb from the parent *C. burnetii* Nine Mile isolate within the chromosomal region associated with synthesis of the O-antigen of LPS [8, 24, 96-98]. There are three different LPS chemotypes of *C. burnetii* that have been reported [24]; Phase I, with full length LPS, Phase II LPS made up of lipid A and some of the core sugars, with the O-antigen sugars are absent [8, 24, 96], and an additional LPS variant named Nine Mile Crazy (NMC) that has an LPS of intermediate in length, that demonstrates intermediate virulence. Interestingly, while the phase II deletion results in a more profound truncation than NMC, NMC has a larger chromosomal deletion, indicating that multiple factors may be involved [99].

While phase transition has been observed in multiple *C. burnetii* strains the classic Nine Mile strain, first isolated in Montana has been the best characterized. In addition, the *C. burnetii* Nine Mile phase II (NMII) isolate that is used throughout the world was clonally purified and represents the only *C. burnetii* strain that can be used outside of BSL3 containment. The phase II truncation of LPS in *C. burnetii* NMII has long been the attribute associated with its avirulence [99]. In addition, the change in surface charge and exposed proteins versus the parent strain enables *C. burnetii* to infect

tissue culture cells at a higher rate. However, after entering a cultured host cell, both *C. burnetii* NMI and NMII traffic, survive, replicate, and interact with the host cell from within the PV their replication within the host cell PV in a phenotypically indistinguishable manner [8, 21, 100-102]. These virtually identical intracellular interactions have made *C. burnetii* NMII an exceptional model with which to conduct host cell-pathogen molecular interaction research.

Antibody titers of IgG to phase II antigens are higher in an acute infection, while chronic infection produce a higher titer against phase I antigen [2, 101, 103-105]. Phase II *C. burnetii* is more susceptible to the membrane attack complex of the complement system [106]. In addition, the full length LPS of phase I *C. burnetii* inhibits antibodies from binding to the bacterial surface proteins while also preventing *C. burnetii* from being detected by Toll like receptors (TLRs) and blocking interaction with the CR3 receptor in dendritic cells and macrophages [100, 107]. Combined, these findings clearly suggest that LPS is closely tied to the ability of *C. burnetii* to cause disease [24, 96, 107].

**Type IVB Secretion System**

As *C. burnetii* is an obligate intracellular bacterium, secretion of virulence determinants into the host cell is crucial. The Type 4 Secretion Systems (T4SS) are a major virulence determinant that enables a number of pathogenic bacteria to deliver effector proteins into their host cell [108, 109]. There are two genetically distinct T4SSs that have been associated with bacterial virulence, the T4A and T4B Secretion Systems [110]. The T4ASS is made up of 11-13 proteins and has been characterized most

extensively in *Agrobacterium tumefaciens* [111]. The system is also found in *Helicobacter pylori* [112], *Bordetella pertussis* [113], *Anaplasma* spp. [114], *Bartonella henslae* [115], *Brucella* spp. [116], and *Rickettsia* spp. [117]. The known T4BSSs possess between 20-26 proteins and have been identified in fewer organisms. There are three bacteria, *Rickettsia grylli, Legionella pneumophila,* and *C. burnetii,* that are known to possess a functional T4BSS [118]. *Coxiella burnetii* utilizes a Type IVB Secretion System (T4BSS), that is a requirement for survival and growth in host cells, and has little homology to the T4ASS [119] The *L. pneumophila* T4BSS is by far the best characterized. Research investigating the genes involved in the intracellular survival and niche environment of *L. pneumophila* identified 20 genes that were necessary for survival, and 6 additional genes that are associated with T4BSS function, but are not essential for intracellular survival [120-122]. These discoveries were made by two independent laboratories, one of which designated the genes *dot* (defect in organelle trafficking) [123] and the other designating them as *icm* (intracellular multiplication), thus the dual naming system in the T4BSS literature [124]. The *L. pneumophila* T4BSS has been shown to be capable of secreting at least 200 effector proteins into the host cell during infection [125].

The first study to propose a loose structural model of the *L. pneumophila* T4BSS was reported in 2006[126]. The authors analyzed immunoblots of cellular fractions of the bacteria to identify the cellular location of Dot/Icm proteins [126, 127]. This study indicated that DotB, DotN, IcmQ, IcmR, IcmS, and IcmW were found in the cytoplasmic fraction, DotA, DotI, DotL, DotM, DotN, DotO, DotP, DotU and IcmF in the inner membrane fraction, DotH and IcmX in the periplasmic fraction, and DotC, DotD, DotF,

DotG, DotH, and DotK in the outer membrane fraction [126]. The immunoblots were unable to determine the location of DotE, DotJ, DotV, IcmT, and IcmV, which were previously predicted to be outer membrane proteins based on Kyte-Doolittle hydrophobicity plots [128]. Protein-protein analysis has suggested that DotC, DotD, DotF, DotG, and DotH interact to form a subcomplex that transverses the cell wall [126, 129]. Additionally, DotL, DotM, and DotN are predicted to form a subcomplex at the inner membrane along with cytoplasmic proteins IcmS and IcmW [130]. The T4BSS has limited homology to other secretion systems, including the T4ASS, and a complete understanding of its molecular structure and function within the bacteria cell envelope does not exist.

A current model of the T4BSS is depicted and summarized in Figure 3 [131]. The *C. burnetii* genome has 23 of the 26 T4BSS genes found in *L. pneumophila*, lacking homologs to the chaperone protein IcmR and predicted inner membrane proteins DotJ and DotV [119, 132, 133]. Studies have demonstrated that *C. burnetii* T4BSS genes are expressed early in infection [134] and that the T4BSS itself appears localized on the pole(s) of the bacteria during infection of host cells [135]. Several T4BSS proteins have been confirmed as being required for intracellular replication. Mutations of DotI [136] and IcmD [137] resulted in a loss of intracellular localization. Targeted deletion of *dotA* and *dotB* showed these genes to also be essential for intracellular growth [138]. Interestingly, *C. burnetii* T4BSS mutants have been shown to be able to replicate within a PV if infections were established in combination with a wild-type *C. burnetii* [137]. This suggests that host cell contact and early infection by *C. burnetii* may not require a

functional T4BSS, but that niche development and subsequent bacterial growth does [136, 139].

There are currently 62 known bacterial proteins that are delivered into the host cell by the *C. burnetii* T4BSS [136, 140-143]. Most of these effector proteins contain eukaryotic motifs, including coiled-coil domains, GTPase domains, ankyrin repeats, leucine rich repeats, and multiple kinases and phosphatases [136, 144, 145]. These eukaryotic motifs are thought to be required for interaction with host cell proteins via protein-protein interactions or regulation of host cell signal transduction pathways [146, 147]. Despite the high degree of T4BSS structural protein homology, *C. burnetii* only encodes six homologs to effector proteins found in *L. pneumophila* [136, 143]. This is likely due to differences in their host environments, the vacuolar niche they require for survival and replication and could even influence the differences in the clinical outcomes of these infections [136, 143].

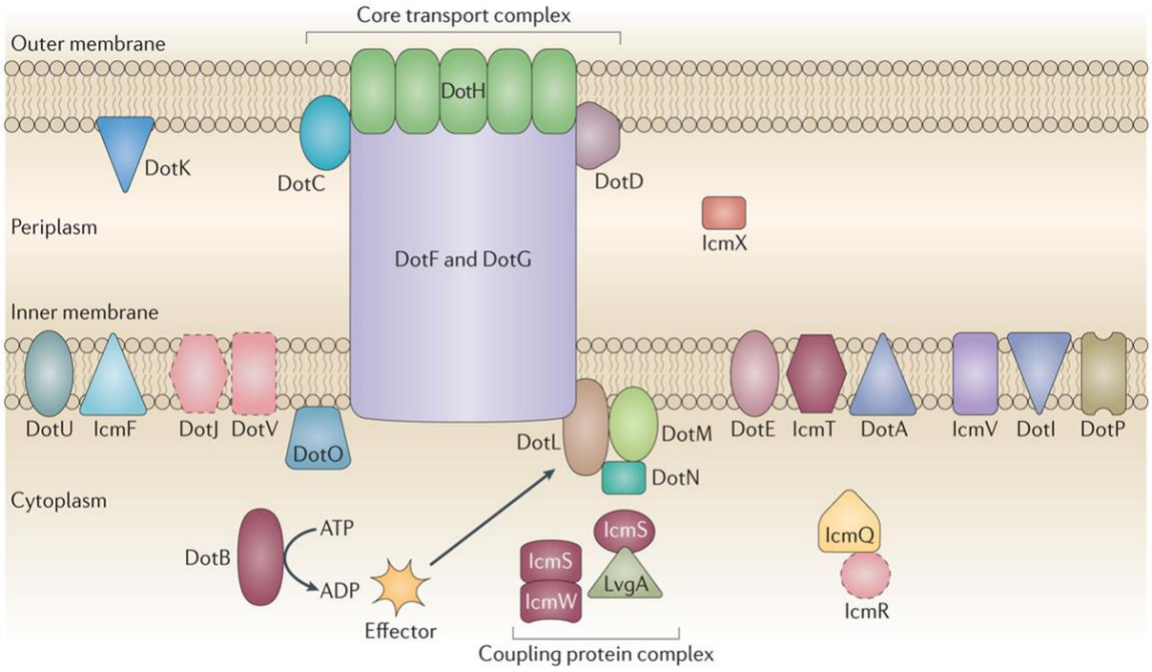


**Figure 3. Model of the Type 4B Secretion System**. T4BSS structural model showing the proposed locations of the Dot/Icm proteins. It is hypothesized that the *C. burnetii* forms complexes similar to that of *L. pneumophilia.* [131].

# CHAPTER III

# INTRANASAL IMMUNIZATION WITH WHOLE-CELL FIXED *COXIELLA BURNETII* NINE MILE PHASE I PROTECTS GUINEA PIGS AGAINST *COXIELLA BURNETII* CHALLENGE

**Abstract**

*Coxiella burnetii* is an obligate intracellular bacterial pathogen that causes Q fever. The debilitating acute form of the disease is characterized by headache, fever, photophobia, and pneumonia while chronic disease is often associated with valvular endocarditis and hepatitis. *C. burnetii* is environmentally stable, has a low infectious dose, is transmitted by aerosol, and was weaponized in the past. While this pathogen is controlled in the general population by pasteurization, it is on the CDC's select agent list because of its ability to cause significant morbidity. Studies have shown that intramuscular, sub-cutaneous, and intraperitoneal administration of whole-cell formalin fixed virulent (phase I) *C. burnetii* vaccine formulations is able to protect subjects from disease following subsequent *C. burnetii* phase I exposure. Our hypothesis was that a whole-cell formulation of killed *C. burnetii* would also be protective when inoculated intranasally. In an effort to determine if an intranasal route of immunization would elicit a protective immune response to *C. burnetii* infection, we used $10_6$ whole-cell formalin fixed *C. burnetii* Nine Mile phase I (NMI) in combination with a double mutant of *E. coli* heat-labile enterotoxin adjuvant in a guinea pig model. Booster immunizations followed at 14 and 28 days. To determine a general measure of the humoral immune response, IgG titers were assayed by indirect fluorescent antibody microscopy using sera collected prior to, and at 14, 28, and 56 days following intranasal immunization. *C. burnetii* NMI specific titers ranging from 4096 to 16384 were detected in day 56 sera, indicating a robust humoral response was elicited using intranasal immunization. In addition, immunized animals subsequently infected with virulent *C. burnetii* NMI did not develop significant clinical symptoms as measured by weight loss or fever. Studies defining minimal immunization requirements, potential hypersensitivity response, and protective

antigen profile using this physiologically relevant exposure route will aid us in defining a protective response to this unusual aerosol acquired pathogen.

## Introduction

*Coxiella burnetii* is an obligate intracellular pathogen and the causative agent of the zoonotic illness Q fever. The acute form of the disease is characterized by headache, fever, photophobia, and pneumonia. Chronic *C. burnetii* infection is often associated with endocarditis and hepatitis [2]. *C. burnetii* has a biphasic life cycle composed of two variants, the environmentally stable small cell variant (SCV) and the replicative large cell variant (LCV) [91]. Transmission occurs by aerosol and has a low infectious dose, with only 1-10 organisms required for infection [9]. This pathogen is controlled in the general population by pasteurization but has been placed on the Center for Disease Control's (CDC's) select agent list because of its low infectious dose and ability to cause significant morbidity [10]. Recent advances in genomics, *in vitro* growth of *C. burnetii*, and the identification of bacterial factors required for pathogenesis offer the promise of identifying novel and effective targets for countermeasure development while advancing our understanding of the its basic biology.

The one currently licensed vaccine, Q-Vax, is only available in Australia. This whole-cell formalin fixed formulation is very effective but can cause a delayed type hypersensitivity response in individuals who have been previously exposed to *C. burnetii.* As a result, everyone must be screened for skin reactivity prior to vaccination [82, 88, 90]. In the U.S., a similarly formulated investigational new drug (IND) vaccine is held by USAMRIID for the vaccination of at-risk research personnel. The cumbersome screening and technical expertise

required to administer these vaccines, however, make them impractical and overly expensive for broad use in military personnel and civilians. With the potential of *C. burnetii* as a weapon or bioterror agent as well as its endemic nature in many developing countries where troops are deployed, the need to understand the basic biology of a protective immune response and to develop an efficacious, safe vaccine against *C. burnetii* that can be administered to anyone is paramount.

While it has been known for years that whole-cell formalin fixed *C. burnetii* provides protection against subsequent infection when administered sub-cutaneously, intramuscularly and even through intraperitoneal injection [2], there appears to be no history of whether this immunization would elicit protection when administered through an intranasal route, thus exposing the mucosa to antigen. Given that *C. burnetii* is acquired through an aerosol route, we sought to determine if whole-cell formalin fixed *C. burnetii* NMI administered along with an adjuvant that has been used recently in rodent intranasal immunization studies [148] would protect guinea pigs against subsequent *C. burnetii* NMI challenge.

**Materials and Methods**

*Bacterial Cultivation and Antigen Preparation***:** Virulent *Coxiella burnetii* Nine Mile Phase I RSA 439 (NMI) was propagated in African green monkey kidney (Vero) cells in RPMI 1640 medium, 5% fetal bovine serum (FBS) at 37°C in an atmosphere of 5% $CO_2$, and the SCV form of the organism was isolated as described previously [95]. Purified *C. burnetii* NMI stocks were enumerated by counting fluorescent forming units (FFU) and stored at -80°C in SPG buffer (0.7 M $C_{12}H_{22}O_{11}$, 3.7 mM $KH_2PO_4$, 6.0 mM $K_2HPO_4$, 0.15 M KCl, 5.0

mM glutamic acid, pH 7.4). For antigen preparation, *C. burnetii* NMI was pelleted at 12,000 g and fixed overnight in 4% formalin at room temperature. Bacteria were then washed in Phosphate Buffered Saline (PBS) (25 mM $NaPO_4$, 150 mM NaCl, pH 7.4) buffer pH 7.4 and also heat inactivated for 30 minutes at 65°C. To demonstrate bacterial inactivation, aliquots were serially diluted and tested for the capacity to infect and replicate in Vero cells. Four days post infection, cells were methanol fixed and analyzed by indirect fluorescent antibody (IFA) microscopy and complete inactivation was confirmed. All *C. burnetii* NMI live growth and manipulations were performed in approved Biological Safety Level 3 (BSL3) laboratory settings.

*Animal Model and Sample/Data Collection***:** Female Hartley Guinea pigs (38-42 days old) were used throughout. Figure 4 outlines the timeline and procedures that occurred within the duplicate trials. Blood draws were performed via the cranial vena cava while weight and body temperature measurements were obtained by digital scale and rectal thermometer. All procedures followed protocols approved by the Oklahoma State University Institutional Animal Care and Use Committee.

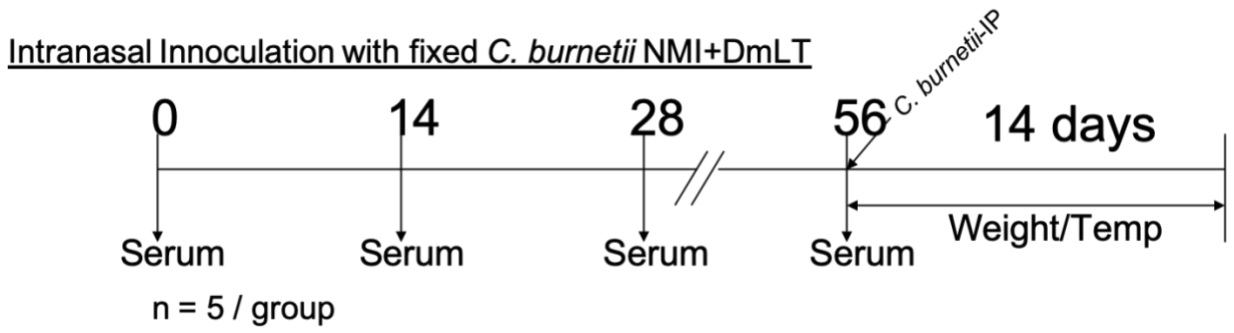


**Figure 4**. **Fixed Whole-Cell *Coxiella burnetii* Intranasal Immunization of Guinea pig**. Immunization, serum collection, *C. burnetii* NMI challenge, and weight/temperature observation(s) days post-challenge are indicated. The same format was used in both trial 1 and 2.

*Immunization***:** Inactivated *C. burnetii* Nine Mile phase I (termed ΔCbI) was diluted in PBS with Double Mutant Lethal Toxin (DmLT) adjuvant (kindly provided by Dr. John Clements, Tulane University) such that 100 μl contained $10_6$ FFU equivalent of *C. burnetii* NMI in ΔCbI form and 2.5 μg DmLT. A 100 μl aliquot of antigen mix was administered to anesthetized guinea pigs (n=5) intranasally via a 1-inch flexible catheter inserted into the nasal canal to achieve deep delivery of the antigen mixture. Animals were given identical booster immunizations at 14 and 28 days following the primary immunization (see Figure 4). Control animals (2 groups of n=5) received sterile PBS mock intranasal immunizations.

*Coxiella burnetii NMI Challenge of Immunized Guinea pigs***:** Live, virulent *C. burnetii* NMI ($10_3$ FFU equivalent) was administered by intraperitoneal (IP) injection [149]

in a volume of 200µl PBS to control and standardize the infectious dose. Intraperitoneal challenge in a guinea pig model is a well-controlled and established means of *C. burnetii* infection [149, 150]. The ΔCbI and one of the PBS immunized groups (mock-immunized controls) received the *C. burnetii* NMI challenge. The other PBS group received sterile PBS IP as a non-challenged control. All animals were monitored daily for weight, temperature, and behavior for a period of 14 days. A pain and distress scoring system (Table 1) was used to determine when animals were under significant stress and required euthanasia [151].

*Indirect Fluorescent Antibody Microscopy***:** Serum was separated from whole blood by centrifugation at 500 g for 5 minutes and subjected to IFA analysis to determine specific IgG maximum-inverse titer. Gamma irradiation inactivated *C. burnetii* NMI (kind gift from Dr. Robert Massung, CDC) and the avirulent *C. burnetii* Nine Mile phase II (NMII) strain were applied to 16-well microscopy slides, air dried, then heat and acetone fixed for use as capture antigen. Two-fold serial dilutions of the Guinea pig sera was used as primary antibody and Alexa-488 goat anti-guinea pig heavy chain IgG (Molecular Probes, Eugene, OR) was used as secondary antibody. Slides were observed at 400X magnification using a Nikon eclipse TE-2000 S inverted microscope.

**Table 1. Pain and Distress Scoring Guide**

## Pain and Distress Scoring Guide

| Score | Details |
|---|---|
| **Standards** | |
| **0.0** | BAR (bright/active/responsive) |
| **0.2** | Quiet but rouses when touched |
| **0.6** | No cage exploration, may vocalize when touched |
| **0.0** | Eupnea (≤150 breaths per minute) |
| **0.2** | Tachypnea (≥150 breaths per minute) |
| **0.6** | Tachypnea and hypernea (≥150 breaths per minute with increased abdominal effort) |
| **0.6** | Bradypnea (<40 breaths per minute) |
| **0.0** | Normal, eating dry food, evidence of urine and feces |
| **0.2** | No evidence of eating food but drinks and appears hydrated (no "tenting" of skin) |
| **1.0** | No interest in food or water and appears dehydrated ("skin tents") |
| **0.2** | Up to 5% weight loss |
| **0.6** | 5-10% weight loss |
| **1.0** | 10-20% weight loss |
| **0.6** | <36.0°C |
| **0.0** | 36.0-40.0°C |
| **0.2** | 40.1°-41.0C |
| **0.6** | >41.0 |

If total score is ≥0.5, observe animal twice per day.
If total score is ≥1.0, euthanize animal.

## Results

*Intranasal Immunization Protects Against C. burnetii NMI Induction of Severe Fever.*

As part of the scoring system used to monitor the pain and distress of the guinea pigs, temperatures were recorded daily (Figure 5). In both trials all mock immunized and mock challenged animals did not record higher temperatures. The animals who received the ΔCbI immunization and were challenged with live virulent *C. burnetii* NMI showed a transient fever, but all began to recover around day 4 following challenge. In trial 1 all of the mock immunized and virulent challenged guinea pigs continued to show an increase in temperature until all were euthanized. In trial 2 all showed an increase in fever until all but one was euthanized. The guinea pig in trial 2 that was not euthanized eventually recovered from the fever.

*Intranasal Immunization Ameliorates Weight Loss Following C. burnetii Challenge.*

Following challenge with *C. burnetii* NMI weights were monitored daily and percent weight change was recorded (Figure 6). The mock immunized and mock challenged guinea pigs did not show a percent loss following the mock challenge. The guinea pigs that received the ΔCbI immunization showed transient weight loss for a few days following challenge in both trials. After 4 days their weights began to trend back upwards. In trial one the guinea pigs who were mock immunized and received the virulent challenge lost weight until they were all euthanized at day 6. In trial 2 they lost weight in the beginning. The one guinea pig that was not euthanized began to regain weight again.

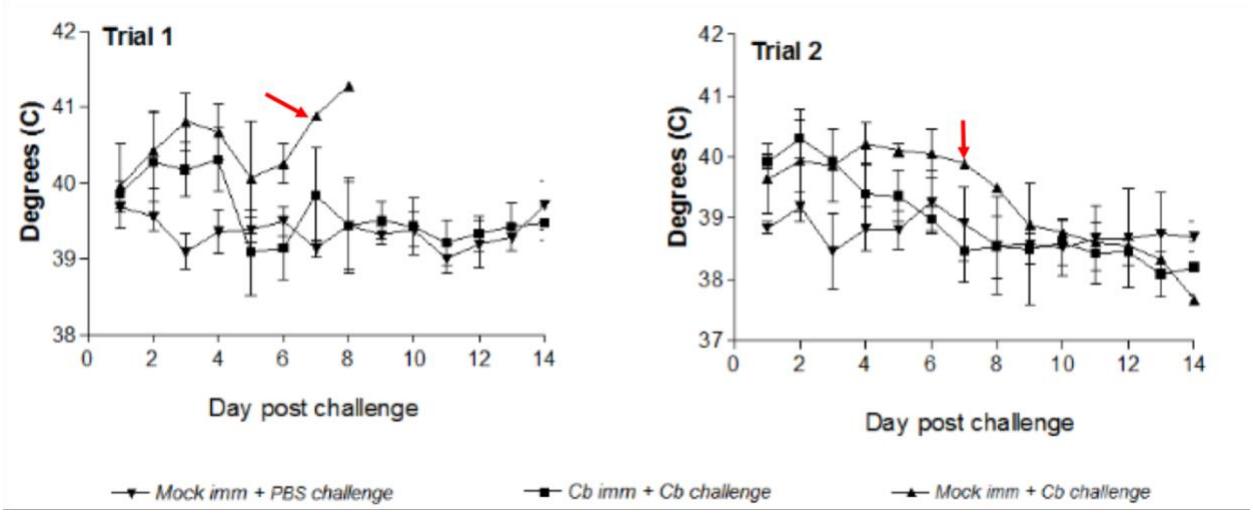


**Figure 5. Temperatures of Guinea Pigs Immunized Intranasally with ΔCbI and Challenged IP 56 Days Later with Live *C. burnetii* NMI.** The mean temperatures of immunized and control groups were compared over 14-days post-challenge. Two identical trials were performed. Each having an immunized group challenged with *C. burnetii* NMI (ΔCbI imm+Cb challenge), a mock immunized group challenged with *C. burnetii* NMI (Mock imm+Cb challenge), and a mock immunized group challenged with PBS (Mock imm+PBS challenge). **A** – Trial 1. **B** – Trial 2. Red arrow indicates the point at which only one Guinea pig of a group survived. * *p value =0.001, unpaired, two-tailed student t-test (Mean ΔCbI imm + Cb v. Mock imm + Cb).*

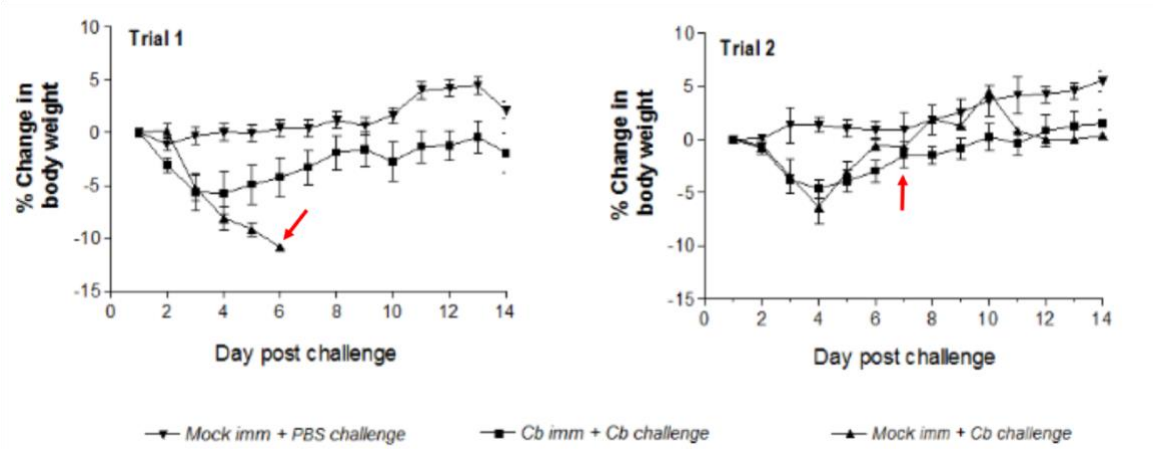


**Figure 6. Body Weight Changes (% total) of Guinea Pigs Immunized Intranasally with ΔCbI and Challenged IP 56 Days Later with Live *C. burnetii* NMI.** The mean % changes in body weight of immunized and control groups were compared over 14-days post-challenge. Two identical trials were performed. Each having an immunized group challenged with *C. burnetii* NMI (ΔCbI imm+Cb challenge), a mock immunized group challenged with *C. burnetii* NMI (Mock imm+Cb challenge), and a mock immunized group challenged with PBS (Mock imm+PBS challenge). **A** – Trial 1. **B** – Trial 2. Red arrow indicates the point at which only one guinea pig of a group survived. ** p value =0.0430, unpaired, two-tailed Student t-test (Mean ΔCbI imm + Cb v. Mock imm + Cb).*

*Intranasal Immunization Increases Guinea Pig Survival Following C. burnetii NMI Challenge.* Following challenge with virulent *C. burnetii* NMI, all guinea pigs were monitored daily and scored based on a pain and distress scoring system (Table 1). When the pain and distress score was 0.5 or higher the observations were increased to twice daily. If the score was greater than or equal to 1.0 the guinea pigs were then euthanized. In both trials

all guinea pigs who received a mock PBS immunization and PBS challenge survived the study. All guinea pigs who received the ΔCbI immunization and were challenged with live *C. burnetii* NMI also survived in both trials (Figure 7). In trial 1 all of the Guinea pigs who received the mock PBS immunization and were later challenged with virulent *C. burnetii* NMI were all euthanized by 6 days following the challenge. In trial 2, one Guinea pig that received the mock immunization and virulent challenge was not euthanized. All others were euthanized by 7 days post challenge.

*Intranasal Immunization Elicits Anti-C. burnetii Antibodies.*

Serum was collected from the guinea pigs prior to immunization and at 14, 28, and 56 days post immunization. Serial dilutions of this sera were used as primary antibody on slides that contained either fixed whole *C. burnetti* NMI, or *C. burnetti* NMII, as antigen. Pre-bleed sera and the sera of the mock immunized guinea pigs had an antibody titer <64 and were considered non-reactive (Table 2). The sera from 14 days post-immunization demonstrated a higher antibody titer to *C. burnetti* NMII than *C. burnetti* NMI. The 28-day antibody titer to *C. burnetti* NMI had increased to a point where it was similar to *C. burnetti* NMII. The 56-day sera had a higher titer to *C. burnetii* NMI than at 28 days and was similar to or higher than the *C. burnetti* NMII titers, which has historically been considered indicative of a more chronic infection [152, 153]. All guinea pigs immunized with ΔCbI had positive antibody titers against both NMI and NMII *C. burnetti*.

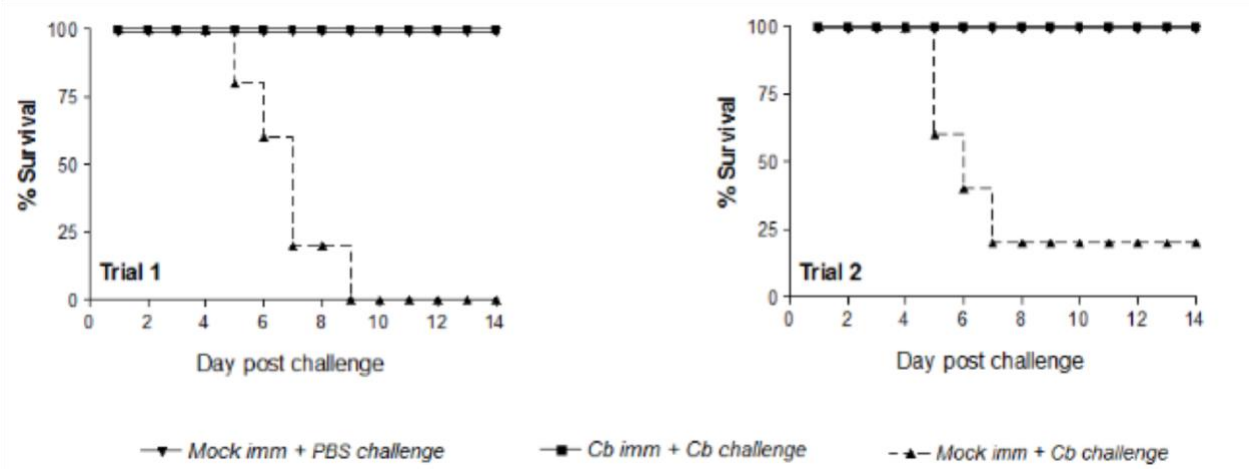


**Figure 7. Survival of Guinea Pigs Following IP Challenge with *C. burnetii* NMI After Previous Intranasal Immunization With ΔCbI**. Immunized and control groups were compared in separate identical trials. ǂ – Nonreactive (NR), indicates the antibody titer was < 64. * - CbI, whole-fixed *C. burnetii* Nine Mile Phase I was used as antigen ** - CbII, whole-fixed *C. burnetii* Nine Mile Phase II was used as antigen. **A** – Trial 1. **B** – Trial 2.

**Table 2. Trial 1 guinea pig reciprocal antibody titer following ΔCbI or PBS intranasal immunization**

| Guinea pig ID | Pre-sera | 14 day sera CbI*/CbII** | 28 day sera CbI/CbII | 56 day sera CbI/CbII |
|---|---|---|---|---|
| ΔCb1 | NR[ł] | 64/16384 | 8192/8192 | 16384/16384 |
| ΔCb2 | NR | 64/16384 | 4096/16384 | 16384/8192 |
| ΔCb3 | NR | 128/8192 | 2048/4096 | 8192/8192 |
| ΔCb4 | NR | 64/8192 | 2048/2048 | 4096/2048 |
| ΔCb5 | NR | 64/8192 | 2048/2048 | 8192/8192 |
| | | | | |
| PBS1 | NR | NR | NR | NR |
| PBS2 | NR | NR | NR | NR |
| PBS3 | NR | NR | NR | NR |
| PBS4 | NR | NR | NR | NR |
| PBS5 | NR | NR | NR | NR |

ł – NR, Nonreactive and indicates the antibody titer was ≤ 64.

* - CbI, Nine Mile Phase I *C. burne*tii was used as antigen

** - CbII Nine Mile Phase II *C. burnetii* was used as antigen

## Discussion

The goal of this study was to evaluate whether intranasal immunization produces a protective response to *C. burnetii* challenge. Guinea pigs were immunized intranasally with ΔCbI + DmLT. At day 56 post initial immunization the animals were challenged with live virulent *C. burnetii* NMI through IP injection to control and standardize the infectious dose delivered [149, 150] (see Figure 4). Temperature (Figure 5), percent change in body weight (Figure 6) and percent survival (Figure 7) were monitored for 14 days post-challenge. In comparing these parameters for ΔCbI and mock immunized animals (both challenged and mock challenged), it was observed that ΔCbI immunized animals had a transient (≈4 day) increase in body temperature following challenge that then returned to control levels while mock immunized animals had elevated body temperatures at a level that contributed to their euthanization (Table 1). Interestingly, one mock-immunized animal that was challenged in Trial 2 did not develop as high of an initial temperature and returned to normal body temperature day 8 post challenge. Measurements of percent body change in weight indicated trends similar to that of the body temperature measurements in the immunized and challenged guinea pigs. The ΔCbI immunized animals had a transient (≈4 day) decrease in body weight, after which time they began to gain weight again. Mock immunized animals that were challenged lost significant weight (Figure 6). The one animal that survived challenge in Trial 2 also began to recover body weight after day 4. All other animals within that group had been euthanized by day 6, thus the survivor altered the weight data averages for this group. Survival analysis (Figure 7) clearly demonstrates the protective effect provided by ΔCbI intranasal immunization. All of the ΔCbI immunized animals survived while all but one animal within the mock immunized groups subsequently challenged with *C.*

*burnetii* NMI required euthanization. It is unclear why the one animal in Trial 2 did not have as significant of symptoms post-challenge. It may have been a technical anomaly or simply the variability in the animal's ability to cope with infection.

Studies along with the existing Q-Vax and IND vaccines have clearly shown that intramuscular, sub-cutaneous, and intraperitoneal administration of whole-cell formalin fixed virulent (phase I) *C. burnetii* strains are able to protect subjects from disease following subsequent *C. burnetii* phase I exposure [2]. However, discovery of the protective epitopes generated from these immunizations has remained elusive. In an effort to determine if an intranasal route of immunization might elicit a protective immune response to *C. burnetii* infection, we used $10_6$ whole-cell formalin fixed *C. burnetii* NMI in combination with DmLT adjuvant, as it has previously been used in intranasal vaccine studies [148]. The use of the intranasal immunization route places antigen(s) in contact with the mucosal immune system, which is likely to generate mucosal, humoral and cell mediated immune responses. In addition, this route may generate immunity through epitopes not recognized by more traditional immunization routes [154]. Previous studies in sensitization by *C. burnetii* immunization indicate the potential for less reactivity when the immunization is delivered intranasally [150]. Further studies of the hypersensitivity response, minimal immunization schedule, alternate adjuvants and the characterization of the immune response elicited through intranasal immunization may help in our quest for an effective and practical vaccine against *C. burnetii*.

# CHAPTER IV

# PROTEIN CHANGES ASSOCIATED WITH DECREASED HOST CELL INFECTIVITY OF *COXIELLA BURNETII* FOLLOWING MULTIPLE PASSAGES IN AXENIC MEDIA

## Introduction

*Coxiella burnetii* is an obligate intracellular bacterium in nature and the causative agent of the zoonotic illness Q fever [2, 12, 15, 131, 155-157]. Upon host cell entry, the *C. burnetii* containing vacuole follows the endocytic pathway, ultimately resembling an altered late endocytic vacuole that is termed the parasitophorus vacuole (PV) [6, 8, 15]. From within this vacuole *C. burnetii* utilizes a Type IVB Secretion System (T4BSS) to deliver effector proteins into the host cell, allowing for survival and pathogenesis [6, 8, 15, 131, 132].

Given its obligate intracellular nature, early *C. burnetii* studies were conducted by maintaining the bacterium through serial passages that required the inoculation of a healthy guinea pig with the blood or minced spleen of an infected guinea pig [1, 2, 16, 17]. In 1939 it was discovered that *C. burnetii* could be cultivated in embryonated chicken eggs, creating a more efficient and standardized means to propagate the organism [17]. Infected yolk sacs could be harvested to produce far greater numbers of *C. burnetii* for subsequent studies [39]. Later, the development and increased usage of eukaryotic tissue culture methods further improved the ability to study *C. burnetii* in more defined techniques as the organism readily infects a multitude of culture cell types [18, 95]. Further advances were made when a metabolism supporting media was developed in 1981that allowed for some short-term physiologic studies, however it did not support *C. burnetii* replication [48]. In the 2000's this metabolic media was used as a starting point from which to develop a fully cell free growth media for *C. burnetii* [19]. In a landmark development within *C. burnetii* research, the first axenic growth media was developed and referred to as Acidified Citrate Cysteine Media (ACCM) [19]. Further improvements to ACCM have been made and include the removal of complex proteinaceous components while increasing bacterial replication, ACCM2 [19,

158], and finally a fully defined media, ACCM-D, which further improved growth while more closely replicating the bi-phasic life cycle of *C. burnetii* that is observed within infected cells [50, 159].

Virulent strains of *C. burnetii* are referred to as phase I *C. burnetii.* When phase I *C. burnetii* are grown continually in eggs, tissue culture, or ACCM, bacteria within the cultures develop chromosomal mutations that lead to a change from phase I to what is called phase II *C. burnetii* [20, 98, 160, 161]. These chromosomal deletions result in a severe truncation of the bacteria's lipopolysaccharide (LPS), causing a subsequent loss of virulence in animal models of infection. These changes were first observed in the original virulent *C. burnetii* Nine Mile isolate (phase I, NMI) after years of passages in embryonated eggs [20]. The avirulent phase II *C. burnetii* Nine Mile (NMII) clonal isolate RSA 493 was ultimately purified from these cultures [162]. This *C. burnetii* NMII strain is recognized as an exceptional model of *C. burnetii*-host cell molecular interactions as it demonstrates intracellular interactions that appear identical to those of the phase I parent strain within tissue culture infections [101, 107, 163]. The genetic mutation that is responsible for the loss of the LPS O-antigen has been frequently noted in *C. burnetii* organisms passaged in a laboratory environment [20, 98, 160, 161]. However, beyond this phase change, it is unclear what pathogen systems and/or genes may change when the environmental stresses found within a eukaryotic host cell are removed.

Here, we hypothesize that *C. burnetii* NMI changes its gene expression within the first few passages upon transition from intracellular to axenic media growth. We used a "reverse evolution" approach to determine potential virulence mechanisms of *C. burnetii* that

are no longer required for intracellular survival as the organism is grown continually in axenic media.

## Materials and Methods

### *Bacterial Cultivation*

*C. burnetii* Nine Mile phase II (NMII) was cultivated in HeLa cells and the SCV form was collected and stored in SPG buffer (0.7 M sucrose, 3.7 mM $KH_2PO_4$, 6.0mM $K_2HPO_4$, 0.15 M KCl, 5.0 mM glutamic acid, pH 7.4) as previously described [95]. Cell propagated *C. burnetii* was used to inoculate 1X ACCM-D (Sunrise Science Products, San Diego, CA) [50, 52] with $10_6$ genome equivalents per mL and grown in a Panasonic $O_2/CO_2$ Incubator for 7 days at 5% $O_2$ and 5% $CO_2$ at 37C. Subsequent passages were created by $10^{-3}$ dilution into freshly prepared ACCM-D and repeated incubation. This methodology was used continually on a 7-day interval to create subsequent passages, as shown in Figure 8.

### *Axenic Media Preparation*

The 1X ACCM-D media was prepared using 2X ACCM-D (-glucose) powder (Sunrise Science Products) and Microbial Cell Culture Grade Water (Fisher BioReagents) following the manufacturer's instructions. After stirring the media for at least 2 hours (longer for larger volumes), the pH was adjusted to 4.75 using NaOH or HCl as appropriate. The pH stability of the media is crucial for subsequent uses. The media was then filter sterilized using either a filter-syringe or vacuum filtration system with a 0.2 um pore size, depending on the

volume being generated. ACCM-D agar plates were prepared using a 2X media preparation of ACCM-D combined with equal volumes of 1.0% agarose (Invitrogen UltraPure™) dissolved in Microbial Cell Culture Grade Water (Fisher BioReagents) as previously published [50].

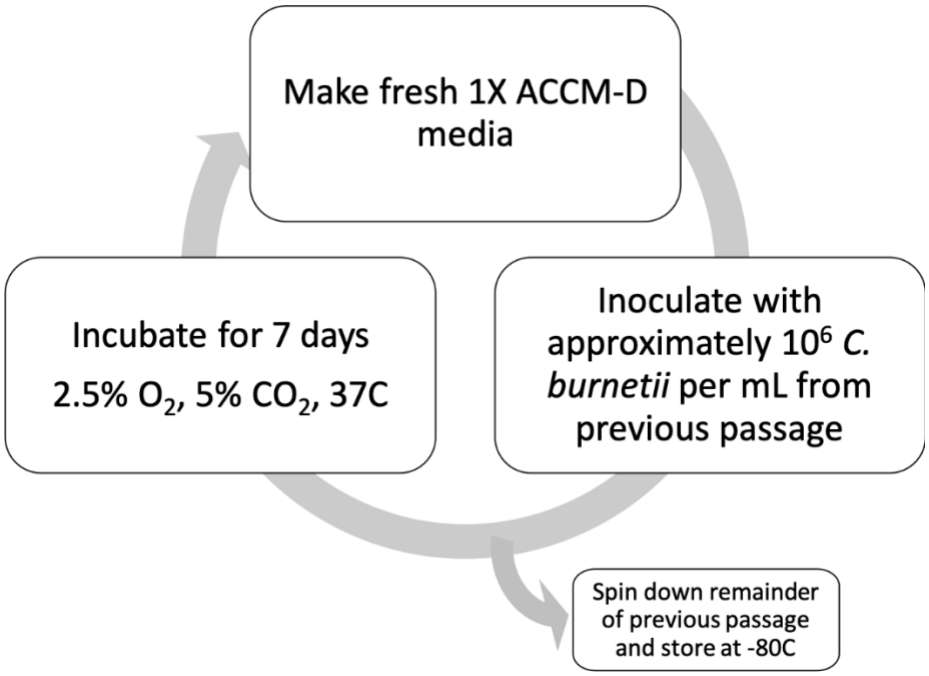


**Figure 8. Passage Methods:** Methods used to passage *C. burnetii* in ACCM-D

*Determination of Genome Equivalents*

Genomic DNA was isolated from samples passaged in ACCM-D by heating 100 uL of the frozen passage sample to 85 C for 10 minutes in 400 uL nuclease free water. These DNA samples were diluted 1:10 and 1 uL of the dilutions were added to SYBR Green Master Mix (Applied Biosystems). Primers to *com1* were used were (forward, 5'-GCACTATTTTTAGCCGGAACCTT – 3' reverse, 5' – TTGAGGAGAAAAACTGGATTGAGA – 3'). Genome standards of $10_6$, $10_5$,$10_4$, and $10_3$ copies were used to create a standard curve. Real-time PCR was performed on an Eppendorf realplex$_2$ Mastercycler and analyzed using the Eppendorf Mastercycler realplex software[134].

*In Vitro Bacterial Enumeration*

To determine colony forming units of ACCM-D cultured *C. burnetii* NMII, ACCM-D agar was initially poured into 6-well tissue culture plates and cooled for 2 hours. Then, various C. *burnetii* NMII passages were diluted in 1X ACCM-D to normalize samples by the number of genomes (genome equivalents) per volume. Subsequent $10_{-1}$ serial dilutions were prepared and 10 uL of each dilution sample was spread plated onto the appropriate ACCM-D agar wells. The inoculated plates were then incubated in a Panasonic $O_2/CO_2$ Tri-gas Incubator at 5% $O_2$ and 5% $CO_2$ at 37C. After 14 days the plates were removed, the colonies were counted, and calculations made to determine the number of *in vitro* viable bacteria in the original sample.

*Host Cell Infectivity and Growth*

To determine the infectivity of serially passaged ACCM-D cultured *C. burnetii* NMII, HeLa cells were seeded onto 96 well culture plates at a density of $10_4$ in RPMI containing 2% FBS 16 hours prior to infection to allow for cellular adherence. Serially passaged *C. burnetii* NMII samples were diluted in RPMI to normalize the number of genomes per volume. Serial dilutions were performed ($10_{-1}$ to $10_{-8}$) and 50 uL of each bacterial dilution was inoculated onto the HeLa cell containing wells and centrifuged at 600Xg for 15 minutes at room temperature [41]. Immediately following centrifugation, the inoculating media was replaced with 200 uL of fresh RPMI containing 2% FBS. The plates were incubated at 37C and 5% $CO_2$. After 72 hours the wells were fixed with ice-cold methanol for 10 minutes in preparation for Indirect Fluorescent Antibody microscopy analysis as previously published [41]. Briefly, *C. burnetii* was stained using rabbit whole anti-*C. burnetii* NMII antibody diluted 1:1000 in PBS containing 3% BSA as a blocking agent. Primary antibodies were detected using Alexa Fluor 488 labeled goat anti-rabbit IgG antibodies diluted 1:1000 in PBS containing 3% BSA (Invitrogen). Total DNA was stained using 4',6-diamidino-2-pheylindole (DAPI) diluted 1:10000 in PBS containing 3% BSA (Molecular Probes) to illuminate host cell nuclei. The methanol fixed and stained cultures were visualized on a Nikon Eclipse TE2000-S and the number of maturing PVs were counted and calculations performed to ascertain the number of fluorescent forming units, which indicates the infectivity of the *C. burnetii* NMII in the ACCM-D samples.

*Immunoblotting*

A 5 mL aliquot of each *C. burnetii* ACCM-D grown passage sample was centrifuged, and the pellet was resuspended in 500 uL Laemmli buffer (BioRad). The protein samples and combined Dual Color and Chemiluminescent Western Standard ladders (BioRad) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Whatman) using a semi-dry transfer apparatus (BioRad) at 12 V for 75 minutes. Membranes were blocked for 1 hour in 5% non-fat powdered milk in PBS with 0.05% Tween-20 (PBS-T) and probed using rabbit anti-sera against either recombinant *C. burnetii* Com1 (diluted 1:10000) (for protein normalizations), DotA (diluted 1:5000) or IcmX (diluted 1:1000) in PBST containing 5% non-fat dry milk as a blocking agent [41]. Following overnight incubation, the membranes were washed three times using PBS-T for 10 minutes. The membranes were then incubated using horse radish peroxidase (HRP) labeled goat anti-rabbit IgG (Cell Signaling) diluted 1:5000 in PBS-T containing 5% non-fat dry milk for one hour at room temperature. Washes were repeated, and the antibody labeled proteins were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Visualization and digital imaging were performed using a ProteinSimple FluorChemE imager. To ensure *C. burnetii* protein content of samples were equivalent, immunoblot analysis and *C. burnetii* total protein was normalized using rabbit anti-Com1 prior to immunoblot analysis of DotA and IcmX [41]. Relative protein quantitation analysis was performed using digital images and Image J [41].

*Mass Spectrometry Analysis*

Cells were lysed in guanidine lysis buffer. Lysates were exchanged into urea, alkylated, and trypsinized using the FASP procedure [164]. Trypsinolytic peptides were further purified by solid phase extraction and injected onto an Acclaim Proteoprep C18 nanocolumn (Thermo PN164642). Columns were developed using a gradient of 3-28 percent water/acetonitrile formulated in 0.1% formic acid, developed over a period of 120 min at 250 nL/min. Eluting peptides were ionized within a Nanospray Flex ion source (Thermo) and analyzed within a quadrupole-Orbitrap Fusion mass spectrometer (Thermo). Precursor ions were analyzed in the Orbitrap sector at nominal resolution 120,000, followed by "Top Speed" data-dependent MS/MS scans using the ion routing multipole for fragmentation by higher energy collisional dissociation and fragment analysis within the ion trap sector. The specific instrument settings are provided within the Supplement.

MaxQuant v1.6.2.10 [165] was used to search instrument RAW files against a database 1,812 *C. burnetii* proteins downloaded from Uniprot on February 6, 2018. Searches utilized default MaxQuant settings, supplemented with Gln cyclization to pyroglutamate as a variable modification and with the "match between runs" feature specified. Protein quantifications were analyzed within the Perseus framework v1.6.7.0 [166] using LFQ protein intensities [167]. A total of three biological replicates were used and student's t-test on log2 LFQ protein intensities were used to determine significance. Protein intensities were considered to be significant with a p-value of $p<0.05$ and a log2 ratio of +/- 0.7.

## Results

### *Coxiella burnetii Infectivity Decreases Following Multiple Passages*

Upon the anecdotal observation that serially passaged *C. burnetii* appeared to infect cultured cells less readily than cell derived bacterial stocks, we wanted to quantitatively assess whether this was the case. Using *C. burnetii* NMII serially passaged 1, 3, 5, and 10 times in ACCM-D, we initiated infections of Hela cells with bacterial dilutions normalized by the number of genomes in each sample. When the number of fluorescence forming units (FFU) per sample were calculated, they revealed a decrease in the number of *C. burnetii* filled vacuoles in tissue culture cells as the bacteria from subsequent passages are analyzed, respectively, resulting in a nearly two-log decrease between Passages 1 and 10 (Figure 9A). This indicates that there are fewer bacteria per genome that are capable of initiating a typical infection following multiple passages.

### *Coxiella burnetii Viability in Axenic Media Does Not Decrease Following Multiple Passages*

We next wanted to determine if the decrease in infectivity of tissue culture cells was associated with a decrease in *in vitro* viability of the *C. burnetii* as measured by colony forming units on ACCM-D agar. To address this question, we plated dilutions of passages 1, 3, 5, and 10 on ACCM-D agar plates and counted the colonies. Contrary to the decrease in infectious units (Figure 9A), the colony counts indicated that there was no significant change in viable bacteria relative to genomes as the organism is serially passaged (Figure 9B). This indicates that the number of live and replicative bacteria is not changing, and therefore not responsible for the decrease in infectivity of cultured eukaryotic observed.

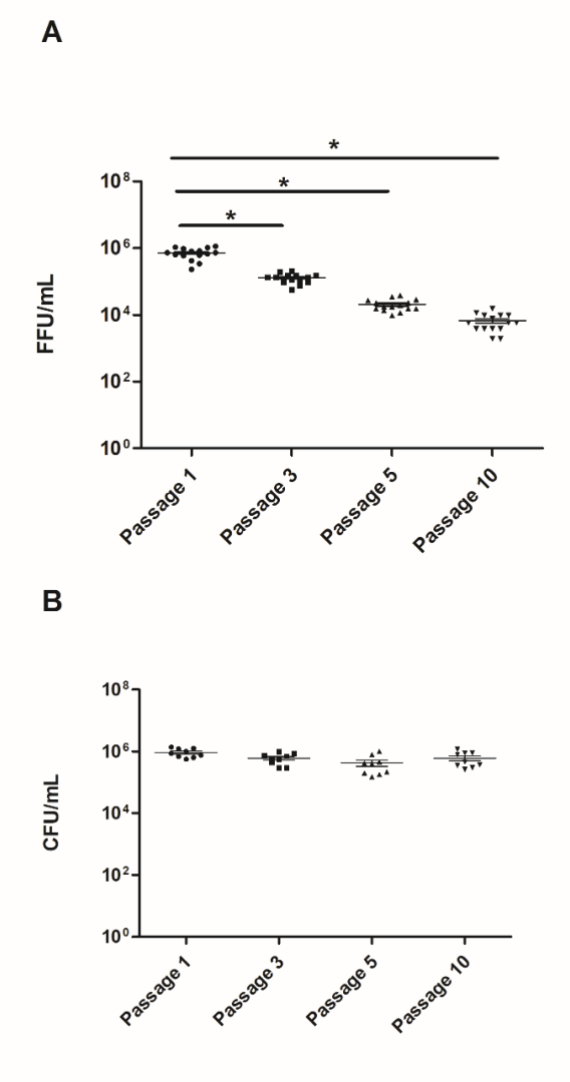


**Figure 9. Intracellular vs Axenic Growth Following Serial Passage of *C. burnetii* NMII**

Intracellular and axenic growth from 3 biological replicates of passaged ACCM growth **A)** FFU counts of infections of HeLa cells normalized by *C. burnetii* genomes from passages 1, 3, 5, and 10. Significance is indicated. *p<0.001. **B)** CFU enumeration of passages 1, 3, 5, and 10 *C. burnetii* spread on ACCM-D plates normalized to genomes. No statistically significant difference was observed between groups.

*Coxiella burnetii T4BSS Proteins Concentrations Decline Following Multiple Passages in Axenic Media*

In an effort to address a possible cause of this decrease in infectivity, we sought to characterize the expression of proteins from a known virulence mechanism, the *C. burnetii* T4BSS. It is well-established that a functional T4BSS is necessary for proper *C. burnetii* vacuole formation and growth of the organism [118, 137, 168]. DotA and IcmX are structural proteins of the *C. burnetii* T4BSS and key components required for its functioning [41, 137]. Immunoblot analysis of *C. burnetii* NMII passages 1, 3, 5, and 10 indicated that protein concentrations of both DotA and IcmX showed very little change in passages 1, 3, and 5. However, a significant decrease of both proteins is observed by passage 10 (Figure 10A). While the decrease in relative DotA concentration is greater than that of IcmX, Figure 10C clearly demonstrated that the expression of both was significantly reduced by passage 10.

To determine whether this was a transient decrease, or if the concentrations of either protein continued to change following continued serial passages. Immunoblot analysis was performed to include passages 15 and 20. These data clearly indicate that DotA and IcmX concentrations in passages 15 and 20 remained consistent with the decreased relative concentrations observed in passage 10 (Figure 10B). These data suggest that sometime after passage 5, the concentration of two T4BSS homologs that are crucial to PV development and *C. burnetii* survival and growth intracellularly decline and remain reduced during subsequent propagation in the ACCM-D growth setting relative to early passages of the organism.

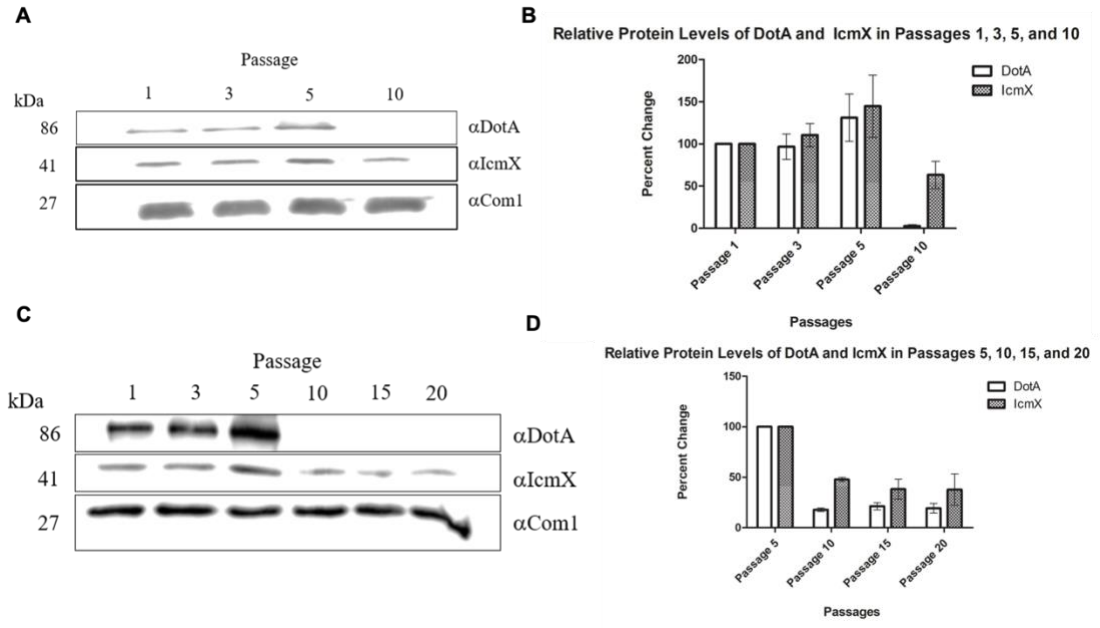


**Figure 10**. **Relative Concentrations of *C. burnetii* DotA and IcmX in Passages 1-20.**

Immunoblot using primary antibody against DotA and IcmX, and the outer membrane protein Com1 as a total protein loading control. **A)** samples from passages 1, 3, 5, and 10. **B)** Bar graph demonstrating protein concentrations of DotA and IcmX relative to Com1 and percent change from passage 1 in passages 1, 3, 5, and 10 on three biological replicates. **C)** samples from passages 1, 3, 5, 10, 15, and 20. **D)** Bar graph demonstrating protein concentrations of DotA and IcmX relative to Com1 and percent change from passage 5 in passages 5, 10, 15, and 20 on three biological replicates.

*Mass Spectrometry Analysis Indicates Significant Proteomic Changes Occur in C. burnetii NMII During Continuous In vitro Growth*

Mass spectrometry analysis was performed on total protein samples of passages 1, 3, and 5 to determine the extent of global proteomic changes occurring during the early serial passages of *C. burnetii* NMII in ACCM-D. Mass spectrometry analysis of passages 1, 3, and

5 revealed several protein expression changes that occurred in these early passages (Figure 11A-C). Proteins with a p-value of $p<0.05$and a log2 ratio of +/- 0.7 were considered to be significantly changed from the passage to which they were compared. The majority of detected protein changes occurred in passage 3 when compared to passage 1 (Figure 11A). Compared to passage 1, there were 109 proteins that were changed in passage 3. Of the 109 proteins that changed, 82 increased in concentration while and the remaining 27 demonstrated decreased concentrations relative to passage 1 (Figure 1D). When comparing passage 5 to passage 1 (Figure 1B), 48 proteins were found in passage 5 to have changed in concentration relative to passage 1. Of these, 20 proteins demonstrated increased concentrations and 28 decreased concentrations. When comparing the proteins that were changed in both passage 3 and passage 5 relative to passage 1, it was determined that 11 proteins were increased in both passages, and 14 proteins were decreased in both (Figure 11D). A comparison of the protein expression of passage 5 relative to that of passage 3, only 4 proteins were found to demonstrate a significant change. Of these, 2 were increases in concentration. Interestingly, one of these was a guanyltransferease that was also increased in passage 5 when compared to passage 1, and the other was an uncharacterized protein that was only determined to be significantly changed between passages 3 and 5. The other 2 proteins demonstrated decreased concentrations. One of these was a membrane protein and the other an enzyme predicted to have lyase activity. It also had a decreased concentration relative to passage 1 (see Appendix).

There were several different types of protein homologs whose concentration increased in passages 3 and 5 relative to that of passage 1. Proteins predicted to be involved in ATP production, membrane synthesis, outer membrane proteins, and transporter proteins

were among those groups with several proteins increasing in concentration. There was also one protein (GrpE) predicted to be involved in protein folding, and 3 proteins predicted to be involved in antibiotic resistance (two multidrug resistance proteins and one beta-lactamase) with increased concentrations. Of particular interest given our host cell infectivity findings, the largest group of characterized proteins whose concentrations were decreased relative to passage 1, were six proteins predicted to be associated with host cell entry. Of these six proteins, two were decreased in both passage 3 and passage 5 when compared to passage 1, and the other four were decreased in passage 5 relative to passage 1 (Table 3). Additionally, there were hypothetical export proteins with reduced expression in both passage 3 and passage 5 relative to passage 1. There were twenty-three uncharacterized proteins identified to be significantly changed. Of these, four were decreased in both passage 3 and passage 5 when compared to passage 1, four were decreased in passage 3 compared to passage 1, and one was decreased in passage 5 compared to passage 1. There were fifteen uncharacterized proteins that were increased; six in passage 3 compared to passage 1, three in passage 3 and passage 5 compared to passage 1, four in passage 5 compared to passage 1, and the one protein uncharacterized protein that was mentioned earlier to be increased in passage 5 when compared to passage 3 (see Appendix). When searching for known T4BSS effector proteins 4 were found to be significantly changed. Of these 4 proteins, one was decreased while the other 3 were increased. This suggests that several protein changes are occurring in these early passages that may further the understanding of proteins necessary for host-cell infection versus those necessary for *in vitro* growth.

**Table 3: Proteins from Groups of Interest that were Found to be Either Increased or Decreased via Mass Spectrometry Analysis**

| Gene Name | Protein | Group | Up/Down | Passages | (-log p-value) | Fold Change |
|---|---|---|---|---|---|---|
| icmP | IcmP | T4BSS | Up | P1 vs P3 | 1.393569145 | 0.798053741 |
| icmG | IcmG | T4BSS | Up | P1 vs P5 | 1.864376676 | 0.800012589 |
| CBU_1244 | Multidrug resistance protein B | Resistance | Up | P1 vs P3 | 1.522278754 | 1.618398666 |
| CBU_0797 | Multidrug resistance protein B | Resistance | Up | P1 vs P3 | 1.88601825 | 1.741046906 |
| ampE | Beta-lactamase induction protein | Resistance | Up | P1 vs P3 | 1.698282239 | 2.319168091 |
| enhA.2 | Enhanced entry protein | Entry | Down | P1 vs P3 and P5 | 3.221317087 | -2.505064011 |
| enhA.1 | Enhanced entry protein | Entry | Down | P1 vs P3 and P5 | 1.896607557 | -1.4284935 |
| enhA.5 | Enhanced entry protein | Entry | Down | P1 vs P5 | 2.43904013 | -3.368894577 |
| enhA.4 | Enhanced entry protein | Entry | Down | P1 vs P5 | 1.787629538 | -3.362092972 |
| enhB.2 | Enhanced entry protein | Entry | Down | P1 vs P5 | 2.011496889 | -2.679271698 |
| CBU_1136 | Enhanced entry protein enhC | Entry | Down | P1 vs P5 | 1.759323242 | -2.183681488 |
| CBU_1984 | Hypothetical exported protein | Exported | Down | P1 vs P3 and P5 | 1.533184984 | -2.561138153 |
| CBU_1843 | Hypothetical exported protein | Exported | Down | P1 vs P3 and P5 | 2.255918541 | -1.277061462 |
| CBU_0110 | Hypothetical exported protein | Exported | Down | P1 vs P3 and P5 | 2.109782192 | -1.242856979 |
| CBU_1135 | Hypothetical exported protein | Exported | Up | P1 vs P3 | 2.247875268 | 0.766370773 |
| CBU_1985 | Hypothetical exported protein | Exported | Up | P1 vs P3 | 2.345103836 | 2.27497673 |
| secG | Protein-export membrane protein SecG | Exported | Up | P1 vs P3 | 2.308438835 | 1.74196434 |

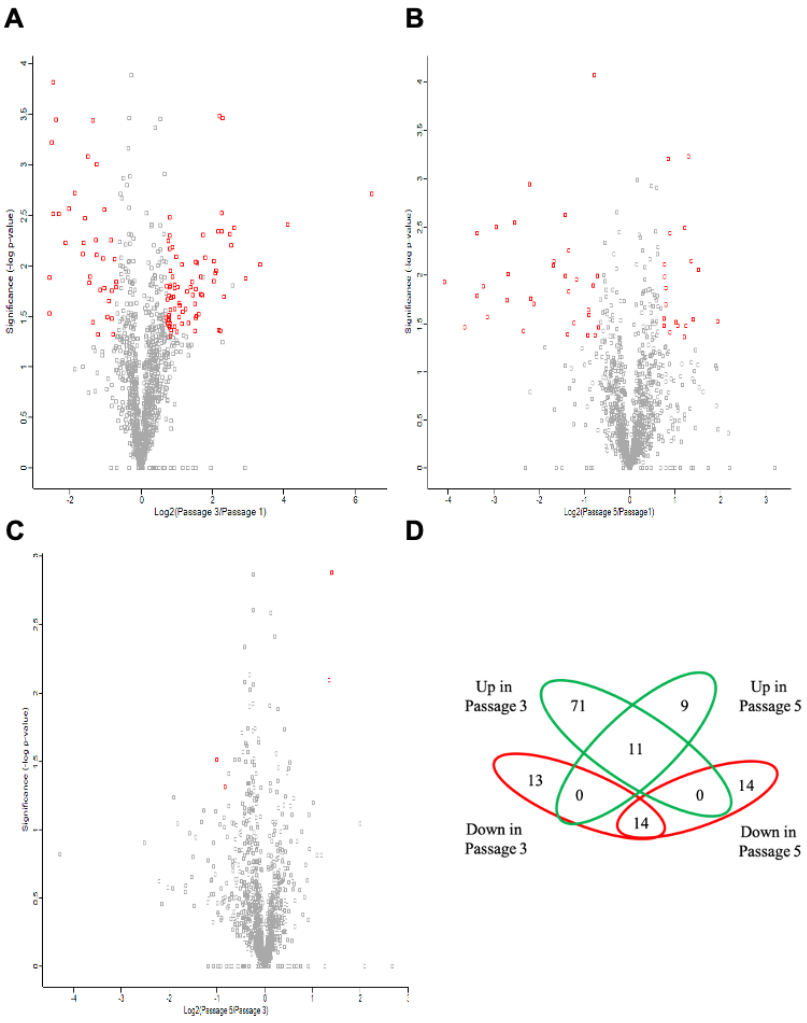


**Figure 11. Analysis of *C. burnetii* Global Protein Content Changes in Passages 1, 3, and 5.** Volcano Plot of log2 transformed protein changes against -log of the p-value from *t*-test in **A)** passage 3 vs passage 1, **B)** passage 5 vs passage 1, and **C)** passage 5 vs passage 3. **D)** A Venn Diagram demonstrating the similarities and differences seen in protein level changes between passage 3 and passage 5 as compared to passage 1. Analysis was performed in Perseus. $p<0.05$ log2 ratio +/- 0.7.

**Discussion**

We hypothesized that repeated passages of *C. burnetii* in ACCM-D would result in a loss of infectivity due to gene expression changes. Propagation in ACCM-D gives *C. burnetii* NMII everything required for growth, yet without many of the obvious environmental stressors present in a eukaryotic host cell [52]. Given the history of phase transition that results in LPS truncation when virulent strains are serially grown in models lacking an immune system, we sought to determine if the reduced environmental stress present during ACCM-D growth would result in phenotypic and molecular changes within *C. burnetii* NMII, and to then begin to determine what those changes may be. Initiating our studies with *C. burnetii* NMII provided us the opportunity to look beyond LPS transition, since the isolate already has that phenotype, and instead look for other uncharacterized changes that may occur when the bacteria are not faced with the struggles of growing in the eukaryotic host cell environment. When the infectivity of bacteria passaged 1, 3, 5 and 10 times were compared, we observed an approximately 2-log decrease in passage 10 compared to passage 1 (Figure 9A). In contrast, colony forming units for the same passages showed no significant differences in *C. burnetii* NMII viability (Figure 9B). Taken together, these data indicate that changes that are occurring during the first 10 passages that are contributing to a decrease in the organisms ability to infect and grow in eukaryotic host cells do not appear to have any effect on the viability of *C. burnetii* NMII while growing in ACCM-D.

In an effort to begin to determine some of the virulence mechanisms that may be involved in the reduction of infectivity, we looked at the expression of homologs of the *C. burnetii* T4BSS, a known virulence mechanism that is required for the formation of the mature PV during infection [137, 138]. Both DotA and IcmX are *C. burnetii* T4BSS

structural proteins [118] and immunoblot analysis of these proteins indicate there is a decrease in protein concentrations by serial passage 10 (Figure 10). While DotA had a greater decrease than IcmX by passage 10, both of these crucial T4BSS proteins are significantly reduced in relative protein concentration relative to earlier passages. To determine whether this decrease was transient, we analyzed the protein concentrations of both in even later passages. Immunoblots clearly demonstrate that passages 15 and 20 had similar protein levels to passage 10 (Figure 10), remaining significantly lower than passages 1,3,and 5. These data suggest that the loss or greatly reduced expression of a functional T4BSS may be one of the molecular changes resulting in a decrease in infectivity and/or PV development following serial passages of *C. burnetii* in axenic media.

Mass spectrometry analysis of the first five passages indicate significant protein expression changes occurring in these early passages (Figure 11). Relative protein concentration reduction in host-cell entry proteins and hypothetical export proteins support our hypothesis that *C. burnetii* proteins no longer needed for growth within a host cell will decrease in these early passages. Interestingly, there were several proteins whose concentration increased that are predicted to be involved in membrane synthesis and structure, as well as ATP synthesis and metabolism. These protein groups and their involvement in these early passages are interesting findings that will lead to future investigation. There were also antibiotic resistance proteins whose concentrations increased. These data collectively indicate that several proteomic changes are occurring in these early passages that have an effect on the passaged bacteria's ability to infect host cells, but do not result in a change in *in vitro* growth. This supports our hypothesis that molecular changes would occur, and the data indicating a decrease in enhanced entry proteins further supports

that these changes are responsible for a decrease in host cell infections. Determining why some of these protein changes are occurring in response to growth in axenic media could help further our understanding of *C. burnetii* virulence and infection. Future studies include comparing our immunoblot findings to passage-matched whole-cell mass spectrometry and RNA seq data, respectively

# CHAPTER V

# SUMMARY REVIEW OF *COXIELLA BURNETII* INTRANASAL IMMUNIZATIONS AND AXENIC MEDIA ADAPTATIONS

It is well established that whole cell killed virulent strains of *C. burnetii* can be used as vaccines, yet they have a myriad of unintended issues. They have been used intramuscularly, intraperitoneally, and subcutaneously to good effect. However, an intranasal route of immunization has never been characterized with regards to protection from *C. burnetii* challenge. We hypothesized that a whole cell killed formulation of phase I *C. burnetii* would also effectively immunize guinea pigs when administered intranasally. Intranasal immunization offers an alternative route that directly exposes the mucosa to the antigen. We tested our hypothesis using a formulation of whole-cell formalin fixed *C. burnetii* combined with the adjuvant DmLT, which has been used recently in other intranasal immunization studies [148]. We discovered that guinea pigs immunized with this formulation were protected when compared to those who received a mock immunization of PBS and DmLT. When challenged with live, virulent *C. burnetii*, those animals that had been immunized showed a modest, transient fever for approximately 4 days, but returned to normal temperature levels thereafter. In contrast, the guinea pigs who received a mock immunization had high fever until euthanization was required. The same proved true of weight loss. Those that were immunized with the killed *C. burnetii* experienced only modest transient weight loss, but soon recovered and regained weight. In the mock immunizations all but one animal continued losing weight until euthanization was required.

Following mock immunization only one guinea pig out of ten did not meet the requirement for euthanization when challenged with virulent *C. burnetii*. Serum from guinea pigs that was drawn following immunization, but prior to challenge, indicated that significant titers of antibodies against both phase I and phase II *C. burnetii* were elicited. The goal of this analysis was to determine if the intranasal route of immunization was protective. We were able to demonstrate that the formulation of whole-cell formalin fixed *C. burnetii* with DmLT was able to protect guinea pigs against virulent *C. burnetii* challenge and that significant antibody titers specifically against *C. burnetii* whole antigen was generated. Future studies would include a

broader characterization of the immune response including determining the immune cells and cytokines involved in the immune response and doing additional antibody titers to IgA to further characterize the mucosal immune response. Additional future studies would include immunization testing on animals with previous exposure to *C. burnetii* to determine the occurrence and severity of the hypersensitivity reaction in intranasal immunizations.

The ability to grow *C. burnetii* in the absence of a host cell is a recent development. Previous laboratory growth outside of animals was performed in embryonated eggs and tissue culture. When grown in embryonated eggs, tissue culture, or ACCM, a well-documented phase transition occurs that results in the development of avirulent phase II *C. burnetii.* However, beyond this phase change, it is unknown what other molecular changes may occur when the stressors of life within a eukaryotic host cell are removed. We had noticed anecdotally that infections initiated with *C. burnetii* NMII that had been passaged multiple times in ACCM-D were not producing an infection level that was expected based on the number of genomes used to initiate the infection relative to infections initiated using host-cell derived bacterial stocks. We know that in nature *C. burnetii* is an obligate intracellular organism [1] and that the development of axenic media is a recent advancement in *C. burnetii* research [19]. While the phase change that occurs when *C. burnetii* is grown outside of an animal host is well understood relative to LPS transition [23-25], we do not know what other molecular changes may occur beyond LPS phase transition when the stressors of an intracellular environment are completely removed.

We hypothesized that physiological changes would occur in the first several passages of phase II *C. burnetii* in ACCM-D resulting from protein expression modulation. To test this, *C. burnetii* NMII was isolated from tissue culture and used to inoculate ACCM-D, after which is was serially passaged weekly in a continuous manner. Within the first 10 passages a significant decrease in the number of vacuoles counted following the infection of tissue culture cells was observed relative to the number of genomes used to initiate the infection. In contrast, the parallel

assays showed no significant difference in the number of colony forming units when the bacteria were plated on ACCM-D agar. This indicates that there are changes in cellular infectivity occurring when intracellular stresses/signals are removed and *C. burnetii* is grown for several generation in cell-free axenic media.

In order to begin elucidating what molecular changes may be contributing to the loss of infectivity, we began by looking at two proteins of the Type IVB Secretion System (T4BSS). The T4BSS is a known virulence mechanism required for productive host cell infection [2], and both DotA and IcmX are structural proteins of the T4BSS. When characterizing DotA and IcmX protein concentrations through the first 10 passages, we found that there were no significant differences between passages 1, 3 and 5, but by passage 10 there was a significant decrease in the amount of both proteins. The decrease in DotA was greater than that of IcmX, with DotA being virtually undetectable by immunoblot in passage 10. To determine if protein concentrations might change in later passages, and to ensure that the decrease in both proteins was not a transient change, we then analyzed the protein concentrations of passages 15 and 20. In both of these passages, DotA and IcmX protein concentrations were not significantly different from those in passage 10, suggesting that for these T4BSS homologs continuous ACCM-D growth downregulates the expression of proteins within this crucial virulence mechanism There were also several protein differences detected in the first 5 passages via mass spec analysis. Some of the proteins that were found to decrease were involved in host-cell entry and hypothetical export proteins. These changes suggest that our hypothesis that proteins that are necessary for host cell infection but may not be necessary for growth in axenic media and may be decreased within early passages may be supported. Interestingly, several proteins including some involved in the bacterial membrane, ATP synthesis, and antibiotic resistance were increased in these early passages. Studies to determine what, if any, effect these changes have on the bacterial cell could help further our understanding of *C. burnetii.* Studies could be done to determine if the increase

in membrane associated proteins and ATP synthesis could be due to an increase in cell size or replication time by doing growth curves or microscopy on the *C. burnetii* from these passages. Might these findings point to an increased capacity of membrane synthesis and ATP production as the bacteria ceases to compete with the host cell and increases its growth rate? Future studies are underway which include mass spectrometry analysis out to passage 10 in order to confirm the significant changes detected via immunoblot analysis and to determine whether other proteins are following the pattern of changing more significantly by passage 10. In addition, RNAseq analysis of passages paired to those analyzed by mass spectrometry is being prepared to determine whether these same gene expression changes are occurring at the transcriptional level. Further immunoblot analysis and *C. burnetii* mutants will be generated in the future to confirm and further characterize changes seen via mass spec and RNAseq data. In the future, longer term passages (> 20 passages) will be analyzed via DNAseq analysis in an effort to identify mutations that occur in genes that are not essential for growth in axenic media but may be necessary for infection As we identify changes that occur during continuous axenic media growth, it is our prediction that we will find mechanisms that are crucial to the intracellular lifestyle and pathogenicity of *C. burnetii* and how it evolved to survive in a most inhospitable environment.

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# APPENDICES

| Gene[c] | Protein[d] | Group[e] | Up/ Down[f] | Passage[g] | (-log p-value)[a] | Fold Change[b] |
|---|---|---|---|---|---|---|
| nuoH | NADH-quinone oxidoreductase subunit H | Aerobic Respiration | Up | P1vP3 | 2.377511 | 2.60862 |
| sodC | Superoxide dismutase [Cu-Zn] | Antioxidant | Down | P1vP3 | 2.258087 | -0.84859 |
| sdhE | FAD assembly factor SdhE | Antibiotic Synthesis | Up | P1vP3 | 1.788635 | 1.38653 |
| sdhE | FAD assembly factor SdhE | Assembly | Up | P1vP5 | 3.235613 | 1.28942 |
| CBU_0178 | Glycine betaine transport ATP-binding protein | ATP | Up | P1vP3 and P1vP5 | 3.203188 | 0.8436 |
| CBU_1638 | ATPase | ATP | Up | P1vP3 | 1.576246 | 0.95437 |
| atpH | ATP synthase subunit delta | ATP | Up | P1vP3 | 1.797322 | 0.71135 |
| CBU_1458 | Hypothetical ATPase | ATP | Up | P1vP3 | 1.493799 | 0.77033 |
| CBU_2021 | Hypothetical ATPase | ATP | Up | P1vP3 | 1.698858 | 0.7794 |
| CBU_1087 | ATP/GTP hydrolase | ATP | Up | P1vP3 | 1.330101 | 0.83297 |
| CBU_1234 | Hypothetical ATPase | ATP | Up | P1vP3 | 1.694969 | 0.90506 |
| atpB | ATP synthase subunit a | ATP | Up | P1vP3 | 1.8757 | 2.92569 |
| gshB | Glutathione synthetase | ATP | Up | P1vP3 | 1.800257 | 0.71206 |
| nuoM | NADH-quinone oxidoreductase chain M | ATP | Up | P1vP3 | 2.080527 | 1.79741 |
| nuoL | NADH-quinone oxidoreductase chain L | ATP | Up | P1vP3 | 1.802325 | 2.56335 |
| groS | 10 kDa chaperonin | Chaperone | Up | P1vP3 | 1.46853 | 0.77878 |
| cydB | Cytochrome d ubiquinol oxidase subunit II | Cytochrome | Up | P1vP3 | 1.952327 | 2.11091 |
| cyoD | Cytochrome O ubiquinol oxidase protein | Cytochrome | Up | P1vP3 | 3.4781684 | 2.20293427 |
| gloB | Hydroxyacylglutathione hydrolase | Detox | Down | P1vP3 | 2.061924 | -0.7345066 |
| CBU_0909 | Sua5/YciO/YrdC/YwlC family protein | DNA | Down | P1vP5 | 1.8960793 | -0.8096237 |
| recJ | Single-stranded-DNA-specific exonuclease | DNA | Down | P1vP5 | 4.0770912 | -0.7825756 |
| ssb | Single-stranded DNA-binding protein | DNA | Up | P1vP3 and P1vP5 | 1.6964315 | 0.80257416 |

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|---|---|---|---|---|---|---|
| ung | Uracil-DNA glycosylase | DNA | Up | P1vP3 and P1vP5 | 2.1825723 | 0.87843513 |
| nusB | Transcription antitermination protein NusB | DNA | Up | P1vP3 | 1.8926087 | 0.89229393 |
| recO | DNA repair protein RecO | DNA | Up | P1vP3 | 1.4951844 | 1.06253624 |
| CBU_0873 | Smr/MutS family protein | DNA | Up | P1vP3 | 1.42651706 | 0.82139969 |
| ku | Non-homologous end joining protein Ku | DNA | Down | P1vP3 | 2.22760569 | -1.6073532 |
| ku | Non-homologous end joining protein Ku | DNA | Down | P1vP5 | 2.25740792 | -1.3443737 |
| mutT | 7,8-dihydro-8-oxoguanine-triphosphatase | DNA | Down | P1vP3 | 1.75800637 | -0.8281517 |
| scvA | Protein ScvA - involved in DNA binding | DNA binding | Down | P1vP3 and P1vP5 | 1.8803008 | -2.565794 |
| enhA.2 | Enhanced entry protein | Entry | Down | P1vP3 and P1vP5 | 3.22131709 | -2.505064 |
| enhA.1 | Enhanced entry protein | Entry | Down | P1vP3 and P1vP5 | 1.89660756 | -1.4284935 |
| enhA.5 | Enhanced entry protein | Entry | Down | P1vP5 | 2.43904013 | -3.3688946 |
| enhA.4 | Enhanced entry protein | Entry | Down | P1vP5 | 1.78762954 | -3.362093 |
| enhB.2 | Enhanced entry protein | Entry | Down | P1vP5 | 2.01149689 | -2.6792717 |
| CBU_1136 | Enhanced entry protein enhC, tetratricopeptide repeat family | Entry | Down | P1vP5 | 1.75932324 | -2.1836815 |
| CBU_1984 | Hypothetical exported protein | Exported | Down | P1vP3 and P1vP5 | 1.53318498 | -2.5611382 |
| CBU_1843 | Hypothetical exported protein | Exported | Down | P1vP3 and P1vP5 | 2.25591854 | -1.2770615 |
| CBU_0110 | Hypothetical exported protein | Exported | Down | P1vP3 and P1vP5 | 2.10978219 | -1.242857 |
| secG | Protein-export membrane protein SecG | Exported | Up | P1vP3 | 2.30843883 | 1.74196434 |
| CBU_1135 | Hypothetical exported protein | Exported | Up | P1vP3 | 2.24787527 | 0.76637077 |
| CBU_1985 | Hypothetical exported protein | Exported | Up | P1vP3 | 2.34510384 | 2.27497673 |
| hemB | Delta-aminolevulinic acid dehydratase | Ion Binding | Up | P1vP3 | 2.48029613 | 0.8051281 |

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|---|---|---|---|---|---|---|
| cyoB | Cytochrome c oxidase polypeptide I | Ion Binding | Up | P1vP3 | 1.34971199 | 1.00341225 |
| CBU_0609 | Mevalonate kinase | Kinase | Down | P1vP3 and P1vP5 | 1.78256113 | -1.0233135 |
| ubiB | Probable protein kinase UbiB | Kinase | Up | P1vP3 | 1.31347036 | 1.19380951 |
| CBU_0789 | Two component system histidine kinase | Kinase | Up | P1vP3 | 1.72695887 | 0.70612907 |
| asnB-1 | Asparagine synthetase (Glutamine-hydrolyzing) | Ligase | Up | P1vP5 | 1.4752231 | 0.75658798 |
| CBU_1954 | (2R)-phospho-3-sulfolactate synthase | Lyase | Down | P1vP5 and P3vP5 | 1.50896499 | -0.9933376 |
| CBU_0718 | Hypothetical membrane associated protein | Membrane | Down | P1vP3 and P1vP5 | 2.22858883 | -2.1329861 |
| CBU_0193 | Hypothetical membrane associated protein | Membrane | Down | P1vP3 | 1.44151615 | -1.3545551 |
| CBU_0980 | Lipoprotein | Membrane | Down | P1vP5 | 1.74191602 | -2.7128868 |
| CBU_1589 | Hypothetical membrane associated protein | Membrane | Down | P1vP5 | 1.82919398 | -1.3464355 |
| CBU_0925 | Membrane-bound lytic murein transglycosylase B | Membrane | Down | P1vP5 | 1.95838756 | -1.1647015 |
| CBU_1058 | Hypothetical membrane spanning protein | Membrane | Down | P1vP5 | 1.99174502 | -0.715395 |
| lapB | Lipopolysaccharide assembly protein B | Membrane | Down | P3vP5 | 1.41238273 | -0.7438679 |
| CBU_1851 | Hypothetical membrane spanning protein | Membrane | Up | P1vP3 and P1vP5 | 1.36015247 | 1.20295143 |
| CBU_1559 | Hypothetical membrane spanning protein | Membrane | Up | P1vP3 and P1vP5 | 1.43316087 | 1.33513641 |
| CBU_1379a | OmpA_membrane domain-containing protein | Membrane | Up | P1vP3 and P1vP5 | 2.52347404 | 2.26925659 |
| CBU_1745 | Membrane endopeptidase, M50 family | Membrane | Up | P1vP3 | 1.89613494 | 1.67358208 |
| CBU_1249 | Integral membrane protein | Membrane | Up | P1vP3 | 1.48709929 | 0.70412064 |
| CBU_0056 | Type I secretion outer membrane protein | Membrane | Up | P1vP3 | 1.80888802 | 0.83018303 |
| bamD | Outer membrane protein assembly factor BamD | Membrane | Up | P1vP3 | 1.6824393 | 0.85115433 |

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|---|---|---|---|---|---|---|
| CBU_0910 | Hypothetical membrane associated protein | Membrane | Up | P1vP3 | 1.63474698 | 1.06691933 |
| CBU_1548 | Hypothetical membrane spanning protein | Membrane | Up | P1vP3 | 1.61044965 | 1.08148575 |
| CBU_1865 | Hypothetical membrane associated protein | Membrane | Up | P1vP3 | 1.54333343 | 1.16347694 |
| lolB | Outer-membrane lipoprotein LolB | Membrane | Up | P1vP3 | 1.74837316 | 1.2867012 |
| ompP1 | Outer membrane protein P1 | Membrane | Up | P1vP3 | 1.35844306 | 1.51163864 |
| CBU_0519 | DedA family protein - cell membrane | Membrane | Up | P1vP3 | 1.4861897 | 1.53298569 |
| CBU_1334 | Hypothetical membrane spanning protein | Membrane | Up | P1vP3 | 2.02921487 | 1.56554604 |
| CBU_0307 | Outer membrane protein | Membrane | Up | P1vP3 | 1.71436723 | 1.71836662 |
| CBU_1814 | Outer membrane protein | Membrane | Up | P1vP3 | 1.36169185 | 2.23165131 |
| omlA | Outer membrane protein assembly factor BamE | Membrane | Up | P1vP3 | 2.31105585 | 2.49395561 |
| CBU_1380 | DoxD-like family | Membrane | Up | P1vP3 | 1.71278167 | 1.46274567 |
| CBU_0419 | Polysaccharide deacetylase family | Metabolism | Down | P1vP3 | 3.44174866 | -2.3920746 |
| fbp | Fructose-1,6-bisphosphate aldolase/phosphatase | Metabolism | Down | P1vP3 | 2.56065209 | -1.0362358 |
| CBU_1269 | Acyl-CoA hydrolase | Metabolism | Up | P1vP3 | 1.79312145 | 0.80471039 |
| CBU_0834 | Methyltransferase | Methylation | Up | P1vP3 | 1.42257144 | 1.1479969 |
| grpE | Protein GrpE | Protein Folding | Up | P1vP3 | 2.29965803 | 0.80470085 |
| CBU_1244 | Multidrug resistance protein B | Resistance | Up | P1vP3 | 1.52227875 | 1.61839867 |
| CBU_0797 | Multidrug resistance protein B | Resistance | Up | P1vP3 | 1.88601825 | 1.74104691 |
| ampE | Beta-lactamase induction protein | Resistance | Up | P1vP3 | 1.69828224 | 2.31916809 |
| CBU_0020 | Ribosome-associated factor Y | Ribosomal | Down | P1vP3 and P1vP5 | 1.76631951 | -1.1515923 |
| rpsL | 30S ribosomal protein S12 | Ribosomal | Down | P1vP3 | 1.32190704 | -1.2042046 |
| rplR | 50S ribosomal protein L18 | Ribosomal | Down | P1vP5 | 1.58337037 | -0.8997498 |
| CBU_0505 | Ribosomal-protein-alanine acetyltransferase | Ribosomal | Up | P1vP3 | 1.6661528 | 0.74196243 |
| rpmA | 50S ribosomal protein L27 | Ribosomal | Up | P1vP3 | 1.39837131 | 0.9522686 |

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|---|---|---|---|---|---|---|
| rsmB | Probable ribosomal RNA small subunit methyltransferase B | Ribosomal | Up | P1vP3 | 3.46153474 | 2.28874588 |
| sspA | Stringent starvation protein A homolog | Starvation | Up | P1vP3 | 1.5621935 | 0.82211304 |
| msrA | Peptide methionine sulfoxide reductase MsrA | Stress Response | Down | P1vP3 and P1vP5 | 1.78672451 | -0.7053413 |
| icmP | IcmP | T4BSS | Up | P1vP3 | 1.39356914 | 0.79805374 |
| icmG | IcmG | T4BSS | Up | P1vP5 | 1.86437668 | 0.80001259 |
| CBU_1361 | Rrf2 family protein | Transcription | Down | P1vP5 | 1.64312238 | -0.9085674 |
| CBU_0943 | Rhodanese-related sulfurtransferase | Transferase | Down | P1vP3 and P1vP5 | 2.46752249 | -1.5703754 |
| CBU_1976 | Mannose-1-phosphate guanyltransferase | Transferase | Up | P1vP5 and P3vP5 | 2.09659877 | 1.35116577 |
| ftsK | DNA translocase FtsK | Translocase | Up | P1vP3 | 1.3623257 | 0.84985542 |
| secY | Protein translocase subunit SecY | Translocase | Up | P1vP3 | 2.00949027 | 3.33418465 |
| lolC | Lipoprotein releasing system transmembrane protein | Transporter | Up | P1vP3 | 2.01189174 | 1.14453697 |
| CBU_0727 | ABC transporter permease protein | Transporter | Up | P1vP3 and P1vP5 | 1.57767805 | 1.25133133 |
| CBU_1808 | Export ABC transporter permease protein | Transporter | Up | P1vP3 | 1.76789706 | 1.53952789 |
| CBU_0504 | Di-/tripeptide transporter | Transporter | Up | P1vP3 | 2.41257718 | 4.10868263 |
| CBU_1573 | Transporter, MFS superfamily | Transporter | Up | P1vP3 | 1.72214916 | 1.6763382 |
| CBU_0959 | Bcr/CflA family efflux transporter | Transporter | Up | P1vP3 | 2.04780116 | 2.04186249 |
| CBU_1646 | Glycine betaine transporter | Transporter | Up | P1vP3 | 1.8525572 | 2.04579163 |
| CBU_0649 | Riboflavin transporter | Transporter | Up | P1vP3 | 2.1991969 | 2.52711105 |
| CBU_0095 | Sodium-calcium exchanger | Transporter | Up | P1vP3 | 1.84448581 | 1.43366051 |
| CBU_0347 | D-xylose-proton symporter | Transporter | Up | P1vP3 | 1.62436911 | 1.50629807 |
| CBU_1796 | Amino acid permease | Transporter | Up | P1vP3 | 2.03790276 | 1.52624512 |
| corB | Magnesium and cobalt efflux protein | Transporter | Up | P1vP3 | 1.50864632 | 1.55477142 |
| CBU_1347 | Glutamate/gamma-aminobutyrate antiporter | Transporter | Up | P1vP3 | 1.36875342 | 2.17575645 |

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|---|---|---|---|---|---|---|
| CBU_1677 | Hypothetical cytosolic protein | Uncharacterized | Down | P1vP3 and P1vP5 | 2.56803039 | -2.0216446 |
| CBU_0921 | Hypothetical cytosolic protein | Uncharacterized | Down | P1vP3 | 1.83336507 | -1.4445572 |
| CBU_1847b | Uncharacterized protein | Uncharacterized | Down | P1vP3 and P1vP5 | 3.81360416 | -2.4774055 |
| CBU_0719 | Uncharacterized protein | Uncharacterized | Down | P1vP3 and P1vP5 | 2.11809452 | -1.6410618 |
| CBU_1930a | Uncharacterized protein | Uncharacterized | Down | P1vP3 | 2.51327735 | -2.4731331 |
| CBU_0562a | Uncharacterized protein | Uncharacterized | Down | P1vP3 | 3.00334899 | -1.254179 |
| CBU_0585 | Uncharacterized protein | Uncharacterized | Down | P1vP3 | 1.49902385 | -0.9471607 |
| CBU_0961 | Uncharacterized protein | Uncharacterized | Down | P1vP3 | 1.32574802 | -0.7779694 |
| CBU_0089a | Uncharacterized protein | Uncharacterized | Down | P1vP5 | 2.10594078 | -1.6824017 |
| CBU_0560 | Hypothetical cytosolic protein | Uncharacterized | Up | P1vP3 | 1.30522275 | 0.81721687 |
| CBU_0018 | Hypothetical cytosolic protein | Uncharacterized | Up | P1vP5 | 1.51360676 | 1.02029228 |
| CBU_1260 | Uncharacterized protein CBU_1260 | Uncharacterized | Up | P1vP3 and P1vP5 | 1.92685132 | 2.07794952 |
| CBU_0049 | Uncharacterized protein | Uncharacterized | Up | P1vP3 | 1.51681145 | 0.7793293 |
| CBU_1820 | Uncharacterized protein | Uncharacterized | Up | P1vP3 | 1.79180022 | 1.02207947 |
| CBU_1224a | Uncharacterized protein | Uncharacterized | Up | P1vP3 | 2.71158259 | 6.46660614 |
| CBU_0835 | Uncharacterized protein | Uncharacterized | Up | P1vP5 | 1.40908311 | 0.88706779 |
| CBU_0964 | Uncharacterized protein | Uncharacterized | Up | P1vP5 | 2.1435972 | 1.36008072 |
| CBU_0827 | Uncharacterized protein | Uncharacterized | Up | P1vP5 | 1.52682563 | 1.94416809 |
| CBU_0827 | Uncharacterized protein | Uncharacterized | Up | P3vP5 | 2.8830768 | 1.40006256 |
| CBU_0937 | UPF0422 protein CBU_0937 | Uncharacterized | Up | P1vP3 and P1vP5 | 2.16990791 | 0.80838776 |
| CBU_1274 | Inhibitor_I42 domain-containing protein | Uncharacterized | Up | P1vP3 and P1vP5 | 1.77710954 | 0.95226479 |
| CBU_1664 | CBS domain containing protein | Uncharacterized | Up | P1vP3 | 1.81143072 | 0.93589783 |
| CBU_1018 | DUF4440 domain-containing protein | Uncharacterized | Up | P1vP3 | 2.33798517 | 2.16584587 |

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|---|---|---|---|---|---|---|
| higA | Virulence-associated protein I | Virulence | Up | P1vP3 and P1vP5 | 2.49118418 | 1.21645546 |

a: (-log p-value) – significance of the associated protein

b: Fold Change – amount of increase or decrease of the associated protein

c: Gene – Gene identifier for the associated protein

d: Protein – Protein Name

e: Group – Assigned group based on protein function

f: Up/Down – indicator of whether the protein was increased (up) or decreased (down)

g: Passage – the passage comparisons in which the protein increase or decrease was observed

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