IDENTIFYING *Coxiella burnetii* GENES ESSENTIAL FOR SUBVERSION OF THE HOST IMMUNE SYSTEM

A Thesis

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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December 2014

Major Subject: Medical Sciences

ABSTRACT

Coxiella burnetii, the causative agent of Q fever in humans, is a Gram-negative intracellular bacterium. Although the organism was first isolated in the 1930s, little is known about the specific mechanisms underlying its virulence. This is largely due to its obligate intracellular lifestyle. Recent advances in both axenic growth and genetic manipulation of *C. burnetii* allowed efficient generation and isolation of random mutations and enabled more definitive studies of the genes essential for virulence. The goal of this project was to generate a large collection of specific isogenic *C. burnetii* mutants and employ *in vitro* and *in vivo* screens to determine the individual contributions of their affected genes to pathogenicity. We used a *Himar1* transposon system to generate a library of 1) defined clonal mutants and 2) pools of random transposon mutants in order to approach saturation with non-lethal mutants of the low virulence *C. burnetii* isolate, Nine Mile, phase II (NMII), RSA 439, which is approved for use in a biosafety level two laboratory. Mutants from both libraries were compared in various growth conditions or infection models to identify differences in growth phenotype relative to wild-type *C. burnetii*. The libraries are also amenable to high-throughput analysis using transposon sequencing or transposon directed insertion site sequencing (TraDIS) to compare pooled mutants between input and output infection assays.

In this study, we optimized methods to generate defined transposon mutants, resulting in mutations in nearly 20% of the predicted open reading frames (ORFs) and provide methodology to expand the library for future studies. Included in these mutants

were a number of bioinformatically predicted virulence factors based on phenotypes in other bacterial pathogens that we further compared in cellular and animal models of infection. Our findings are consistent with previous studies that demonstrate the Dot/Icm T4BSS is essential for generating and replicating in a large parasitophorous vacuole (PV). We developed methods for high throughput screening of Tn mutants *in vitro* by imaging on either confocal microscope or BioTek Cytation3 imaging system. We generated a genome saturation transposon mutant pool by combining transposon mutant pools from 35 independent transformation reactions. These combined *in vitro* and *in vivo* screens dramatically improve our knowledge of specific virulence determinants for this pathogen and provide a substantial amount of data for future studies.

DEDICATION

To my husband, James. Your love and support give me strength.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Samuel, and my committee members, Dr. de Figueiredo, Dr. McMurray, and Dr. Skare, for your guidance and support throughout the course of this research.

I am very grateful to Erin van Schaik for her guidance and helpful scientific discussions, Elizabeth Case for a thorough review of this document, members of the Samuel lab for their friendship and support, and the MPIM department faculty and staff for making sure all we have to worry about is our research.

This work was supported by funding to James E. Samuel from NIH 1RO1A1090142-01A1 and DTRA HDTRA1-13-1-0003.

NOMENCLATURE

CCV	*Coxiella* Containing Vacuole
CDC	Centers for Disease Control and Prevention
Cm	Chloramphenicol
HITS	High-throughput Insertion Tracking by deep Sequencing
Kan	Kanamycin
LPS	lipopolysaccharide
NMC	*Coxiella burnetii* strain RSA514, Nine Mile Crazy Phase I/II
NMI	*Coxiella burnetii* strain RSA493, Nine Mile Phase I
NMII	*Coxiella burnetii* strain RSA439, Nine Mile Phase II
ORF	Open Reading Frame
PV	Parasitophorous Vacuole
T1SS	Type 1 Secretion System
T2SS	Type 2 Secretion System
T4BSS	Type IVB Secretion System
T4P	Type IV Pilus
Tn	Transposon
TraDIS	Transposon directed insertion site sequencing

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Background

Coxiella burnetii is a Gram-negative, intracellular bacterium that causes acute and chronic Q fever in humans. It is highly infectious, requiring very few organisms to cause disease. Infection in most animals is asymptomatic but causes abortion and infertility in domestic animals including cattle, sheep, and goats [1]. Acute Q fever is a self-limiting illness in humans typically presenting as a flu-like syndrome, pneumonia, or granulomatous hepatitis with symptoms developing within a few days to a few weeks after exposure. The acute disease is effectively treated by antibiotics, which reduce the duration of symptoms and likelihood of chronic infection [2]. Still, many people never receive treatment because they do not show symptoms of the disease. While most patients recover from the acute infection, others can develop severe, chronic disease most often presenting as endocarditis. Chronic infection, which is fatal in some cases, may develop weeks or even years after the acute infection. The disease is distributed worldwide, with 100-200 cases reported annually in the United States [3].

Due to its non-specific disease presentation, *C. burnetii* infection can be difficult to distinguish from other diseases, making early detection a challenge. While antibiotic therapy is most effective if started within a few days of infection, antibodies cannot be detected until a week or two after infection. Physicians must therefore rely on clinical suspicion when diagnosing Q fever. This diagnosis can later be confirmed by an indirect

immunofluorescence assay (IFA), a serological test for reactivity of serum with fixed whole cell *C. burnetii*. Antibiotic treatment with doxycycline is most effective and should be started early, even before lab results confirm the disease [4].

C. burnetii can survive in extreme weather and environmental conditions for long periods of time and can resist many common disinfectants. Humans typically become infected by inhaling contaminated dust particles. Personnel working with livestock and in research laboratories are considered high risk as infection commonly occurs via the respiratory route following inhalation of infectious aerosols produced by these animals. Infection by ingestion and human-to-human transmission are possible, but rare [5].

Genome sequencing indicates the *Coxiella* genome ranges between ~2.0 – 2.2 Mb and are organized as circular chromosomes normally associated with a single autonomous plasmid [6]. *C. burnetii* isolates were analyzed and differentiated into six distinct genomic groups (I to VI) based on DNA restriction fingerprints [7]. Human isolates within groups I, II, and III were derived from acute disease patients, while isolates in groups IV and V were from chronic disease patients. Isolates in group VI were isolated from rodents near Dugway, Utah [7]. Four plasmid types have been designated, termed QpH1, QpRS, QpDV, and QpDG [8,9]. These plasmids share a common 25-kb region. Strains that lack plasmids contain chromosomally integrated plasmid-like sequences (IPS), suggesting plasmid and IPS genes are important for infection [10].

Early studies showed a correlation between the plasmids and a distinct genomic group. However, since nucleotide sequences were determined for these plasmids [6] and

genotyping revealed crossover between plasmids with different groups [11], human disease may not be determined by plasmid type. However, because these sequences are maintained by all *C. burnetii* isolates, they are believed to be critical for survival [10]. Gene designations distinguish between chromosome and plasmid sequences. Three letters (CBU) followed by four numbers designate genes located on the chromosome, while four letters (CBUA) followed by four numbers designate genes on the plasmid.

The *Coxiella* strain used in this study was isolated from a tick in 1935 collected in Nine Mile Creek, Montana, and therefore designated the Nine Mile (NM) strain. A group studying Rocky Mountain spotted fever discovered this unknown agent about the same time the first outbreak of Q fever occurred in Australia [12]. A laboratory-acquired infection led to the determination that the Q fever and Nine Mile agents were the same pathogen [4]. The NM strain is in chromosomal group I and contains the cryptic QpH1 plasmid. *C. burnetii* plasmids and IPS have been shown to encode Dot/Icm T4SS substrates, including three that are specific to QpH1 [10]. Generating mutants in these genes will be important in defining their roles in infection and virulence.

Unlike many other bacterial pathogens, *C. burnetii* requires a eukaryotic host vacuole for metabolism and replication. This presents a particularly unique problem for the host immune system, since the bacterium actually resides in macrophages, the cells responsible for killing invading pathogens [13]. *C. burnetii* lipopolysaccharide (LPS), studied for its potential role in virulence, undergoes phase variation in the laboratory setting from virulent or “phase I” (PI or NMI) to avirulent or “phase II” (PII or NMII). Virulent *C. burnetii* produces a full-length, smooth LPS with a complete O-antigen that

becomes truncated after serial passage *in vitro*. PII *Coxiella* have rough LPS, which lacks O-antigen sugars, and some LPS core components [5]. A second LPS variant of NMI, Nine Mile Crazy (NMC), has been described as producing intermediate-length LPS and having intermediate virulence [14]. As the O-antigen is the only known alteration between virulent and avirulent *C. burnetii*, PI-LPS is hypothesized to play a critical role in virulence. Comparisons of disease caused by PI and PII *C. burnetii* in SCID mice demonstrated that acquired immunity is essential to overcome both infections [15].

While there are control measures that can be taken to prevent the spread of Q fever, immunization is the most practical method for preventing the disease in both animals and at-risk humans. A PI inactivated whole cell vaccine, derived from the Nine Mile *C. burnetii* strain (Coxevac, CEVA, France) has proven effective for immunizing ruminants [16]. The only human vaccine currently in use is a formalin-inactivated *C. burnetii* PI whole cell vaccine, Q-VAX®, derived from the purified LPS of the Henzerling strain (Commonwealth Serum Laboratories, Parkville, Victoria, Australia), which is produced and licensed for use in Australia. Neither of these vaccines is approved for use in the United States [17].

Though the protective efficacy of Q-VAX® approaches 100%, this vaccine causes severe reactions in individuals who have previously been exposed to *C. burnetii* [18]. A number of individuals who have not previously had contact with the bacterium experience less severe, but still serious side effects. Therefore, this vaccine requires costly, time intensive, and unreliable prescreening prior to administration [19].

Consequently, there is a need for a safe and effective subunit vaccine for human use against *C. burnetii*.

Understanding the mechanisms of protection induced by the PI vaccine would be valuable in developing a safe and effective new generation vaccine against Q fever. Components of Q-VAX® that may allow it to be an effective vaccine include PI LPS or a component of it, proteins that stimulate the cell-mediated immune (CMI) response or antibody-mediated immune (AMI) response, or possibly the slow biodegradability of the small cell variants of *C. burnetii* [20]. A study evaluating the protective activity of PI-LPS and PII-LPS suggests PI-LPS might be responsible for PI vaccine-induced protection against *C. burnetii* infection [5]. Both AMI, or humoral, and CMI responses are believed to be important for developing protective immunity against *C. burnetii* infection [21]. This was demonstrated in a study examining PI vaccinated mice. Passive transfer of immune sera provided significant protection against *C. burnetii* infection. Similarly, bacterial loads were significantly reduced in mice receiving immune splenocyte and T cells [5]. Future Q fever vaccine approaches should focus on boosting both the AMI and CMI responses.

Between 2007 and 2011 in the Netherlands, a Q fever outbreak involved more than 3500 human cases, leading to 24 deaths [22]. Most cases occurred in the spring and early summer and were concentrated in the south of the country. A vaccination program was put into effect when dairy goats were identified as the source of the human Q fever cases, and farmers had to vaccinate their animals whether infected or not [23]. More drastic veterinary measures were taken when vaccination did not successfully contain the

outbreak. Between December 2009 and June 2010, over 50,000 animals were culled on infected farms [24]. This outbreak is a reminder that Q fever remains a threat worthy of continued research to improve our understanding and overcome challenges with diagnosis and treatment.

Its highly infectious nature, aerosol transmission, and ability to survive in adverse environments make *C. burnetii* a potential biological weapon and have led to its classification by the Centers for Disease Control and Prevention (CDC) as a Category B select agent [25]. A biological warfare attack with Q fever would cause a disease similar to that occurring naturally. Though the disease is rarely fatal, it would put a significant strain on our healthcare system. If used in a cocktail with a Category A agent, Q fever would certainly complicate the response to an outbreak. In addition to its relevance as a potential bioterrorism agent, *C. burnetii* also presents a risk for natural infection in deployed military personnel. There have been greater than 30 cases reported among US service members deployed to Iraq and Afghanistan, and the disease continues to be a global threat to both humans and livestock [26].

Virulence Model

C. burnetii has a biphasic lifecycle and is able to transition between two developmental stages that contribute to its environmental stability. Small cell variants (SCV) are typically rod shaped, 0.2-0.5 μm long, infectious particles that are metabolically inactive [27]. SCVs exist in the extracellular environment and are resistant to harsh environmental conditions [1]. Once inside the host cell, the SCV transitions to a large cell variant (LCV), which is metabolically active and can exceed 1 μm in length

[27]. This shift from SCV to LCV occurs alongside maturation and acidification of the PV, without replication, and accounts for the initial lag phase in *C. burnetii* growth [27]. LCVs replicate for many days, expanding the PV. Once large numbers of LCVs accumulate, *C. burnetii* converts back to SCVs which are then released by a still unknown mechanism [28]. Proteins expressed between these two cell variants could potentially be virulence factors. A comparison of protein profiles between the two variants found 48 proteins that were greater than two-fold more abundant in LCVs than SCVs and six proteins that were at least two-fold more abundant in SCVs [25]. These proteins could potentially be involved in immune evasion and should be studied further.

Once inside the host cell, intracellular pathogens typically employ mechanisms to subvert phagosomal maturation. *Mycobacterium tuberculosis,* for example, arrests phagosome maturation at an early stage. *Legionella pneumophila* promotes fusion of the *Legionella* containing vacuole with endoplasmic reticulum-derived membranes instead of endolysosomal compartments, and *Listeria monocytogenes* escapes the phagosome and replicates in the cytoplasm [29]. *C. burnetii,* however, actively directs the maturation of a phagolysosome-like compartment known as the *Coxiella*-containing vacuole (CCV) [30]. This parasitophorous vacuole (PV) has lysosomal characteristics, containing LAMP-1 and cathepsin D [31]. *C. burnetii* actively modifies this PV, but the specific proteins involved are still being identified. Recently, T4SS effectors were shown to be secreted through the membrane of the PV, a requirement for *C. burnetii* maturation and replication [32]. Additional work is needed to determine potential virulence factors involved in PV development and *C. burnetii* replication inside this harsh environment.

Virulence Determinants

Bacterial virulence factors are expressed proteins or toxins that enable the organism to enter, replicate, and persist in a host. They are typically defined by comparing mutated strains to wild type and looking for avirluent phenotypes. This hypothesis is based on Koch's postulates, which establish criteria to determine if a specific microbe causes a disease [33]. Currently, only two virulence factors have been confirmed for *C. burnetii:* LPS and a type IVb secretion system [34,35]. The identification of additional *C. burnetii* virulence factors using recently developed genetic techniques will be instrumental in finding targets for new therapies. Several putative *C. burnetii* virulence factors will be discussed here.

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria and is exposed on the cell surface of unencapsulated bacteria. Lipopolysaccharides are made up of three structural components: lipid A, a hydrophobic lipid section responsible for its endotoxic properties; a hydrophilic inner and outer core polysaccharide chains; and a species-specific O-antigen oligosaccharide side chain [36]. LPS phase variation is a shift from virulent phase I cells containing smooth, full-length LPS I to avirulent, rough phase II cells with a modified LPS II that lacks the O-antigen. This occurs after passage of *Coxiella* in a non-immunologically competent host. In contrast to other bacterial pathogens, phase variation in *C. burnetii* phase II is irreversible due to the large chromosomal deletion associated with the rough phenotype [37]. The deletion includes 21 ORFs (CBU_0679 to CBU_0698); however, there are

likely additional mutations since NMII has a shorter deletion but produces a more truncated LPS than NMC [38].

NMI LPS is hypothesized to shield potential TLR ligands, such as lipoproteins, from recognition. NMI bacteria can persist in DCs without activating them *in vitro*. Infection of DCs with the avirulent NMII strain, however, induces a robust immune response that efficiently clears the bacteria. [34]. This is interesting given the primary function of a dendritic cell (DC) is to alert the immune system, not clear invading pathogens.

Adherence is the first major interaction between an intracellular pathogen and its host and is a prerequisite for bacterial pathogenesis. Adhesins mediate adherence to the host cell by exploiting and binding to cell surface receptors typically used for normal cell processes. Integrins are a group of eukaryotic cell surface receptors that have been shown to be used by a number of pathogens for adherence [39]. While internalization of *C. burnetii* by the host cell requires attachment, the specifics of this interaction remain to be determined. The current understanding is that virulent *C. burnetii* bind monocytes and macrophages, their primary target cells, using leukocyte response integrin ($\alpha_v\beta_3$ integrin) as the dominant receptor and CR3 ($\alpha_M\beta_2$ integrin) as a secondary receptor for NMII [40]. The same study showed avirulent phase II *C. burnetii* are more efficiently phagocytosed than virulent organisms. However, this could result from a more hydrophobic membrane on phase II organisms lacking O-antigen. The effects of these receptors on virulence and the adhesion for $\alpha_v\beta_3$ are yet to be determined [41].

A number of different cellular responses are triggered when *C. burnetii* binds host cells, but their involvement in the establishment and/or clearance of an infection is unknown. *C. burnetii* induces macrophages to produce TNF-α and IL-1α*;* however, a different response is seen in phase I and phase II organisms. Phase I induces greater TNF-α production early in infection, while secretion of TNF-α occurs later with phase II [42]. Also, TNF-α production is specific to phase I in dendritic cells [34] , as is IL-1α production in macrophages [42]. Phase I *C. burnetii* induce actin cytoskeleton reorganization and membrane ruffling when bound to host cells, a response not seen with phase II bacteria. This is intriguing because activation of the protein tyrosine kinase (PTK) inhibitors that causes membrane ruffling negatively regulates bacterial phagocytosis [43]. Further studies are necessary to understand exactly how the differences in phase I and phase II *C. burnetii* contribute to their distinct pathogenesis.

Toll-Like Receptors (TLRs) are an important innate host defense against microbial infection. TLRs recognize structural motifs known as pathogen-associated molecular patterns (PAMPs), expressed by pathogens, or danger-associated molecular patterns (DAMPs), released by necrotic or dying cells [44]. Of the ten human and twelve murine TLRs characterized, two have been shown to be activated by *C. burnetii* PAMPs: TLR 2, which typically recognizes Gram-positive bacteria, and TLR 4, which is predominantly activated by LPS [34,45,46]. Unfortunately, the findings of these studies are not in agreement. Results from one study, for example, suggest that interaction of phase I *C. burnetii* interacting with TLR 4 induces uptake [46], although it was shown that *C. burnetii* LPS acts as a TLR 4 antagonist [45]. How *C. burnetii* interfaces with the

immune system is important to understand the dynamics of infection, and additional studies should be conducted to better characterize the interactions that occur between *C. burnetii* and host cell receptors.

The second major virulence factor that has been identified for *C. burnetii* is the Type IVb Secretion System (T4BSS). It is essential for the formation of the spacious parasitophorous vacuole (PV) required for intracellular replication of *C. burnetii*. This T4BSS is conserved with the Dot/Icm secretion system of *Legionella pneumophila*, encoding 23 of the 26 Dot/Icm homologs [47]. This system will be discussed in detail in the following section.

There is some evidence that the *Coxiella* T4BSS has a role in subverting the host autophagic pathway. Autophagy is a pathway whereby cytoplasmic components are sequestered within a double-membraned compartment (the autophagosome) and degraded upon lysosomal fusion. Generally, autophagy is employed to maintain cellular homeostasis in eukaryotic cells; however, it also assists in the eradication of invading pathogens. Many intracellular bacterial pathogens, including *C. burnetii*, subvert or modulate the autophagic pathway to maintain a replicative niche [48]. While other intracellular pathogens lyse and escape from the phagosome or modify the phagosomal compartment, *C. burnetii* is the only bacterial pathogen known to actually survive and replicate in the acidic, degradative PV [49].

One study hypothesizes that *C. burnetii* exploits the autophagic pathway to delay fusion with lysosomes, allowing the organism to efficiently replicate inside the host cell [50]. Proteins implicated in this process include host Beclin 1 and Bcl-2 [51] , and the *C.*

burnetii T4BSS substrates AnkG and Cig2 [52,53]. Interaction between the autophagic protein Beclin 1 and the anti-apoptotic mitochondrial protein Bcl-2 induces anti-apoptotic activity. This suggests a link between *C. burnetii's* interaction with autophagosomes and its anti-apoptotic activity [51] (Figure 1).

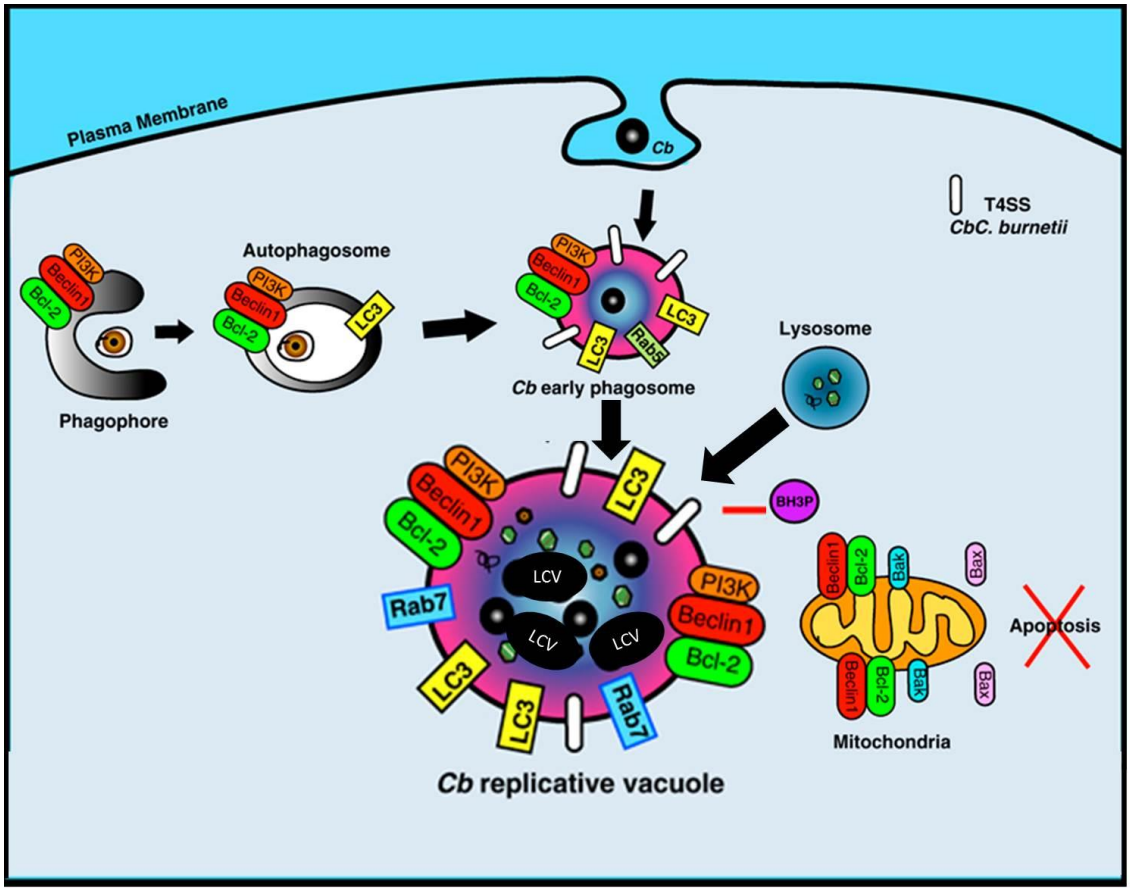

Figure 1: Modulation of the autophagy and apoptosis pathways by *C. burnetii*. (modified from Vazquez and Colombo [51]). Once *Cb* is phagocytosed into the host cell, it uses a T4BSS to secrete proteins which induce the autophagy pathway. The compartment containing *Cb* fuses with autophagosomes, acquiring autophagic key proteins such as LC3, Rab24 and Beclin 1, which favor the generation of the large replicative vacuoles in which *Cb* survives and multiples. *Cb* recruits the anti-apoptotic protein Bcl-2 and inhibits apoptosis to establish a persistent infection.

C. burnetii induces apoptosis through the release of cytochrome c. Prevention of apoptosis could be used by *C. burnetii* to cause persistent infection, while induction of apoptosis later on would enable the infection to spread [54]. The T4BSS effector protein AnkG has been described to be involved in apoptosis by its interaction with host protein p32 [52]. Two other very recent studies implicate the Dot/Icm T4SS and its effectors in autophagy subversion and the recruitment and fusion of autophagosomes during *C. burnetii* infection [53,55]. Continued studies will identify and characterize individual effectors involved in and specific pathogenic mechanisms directing *C. burnetii* interaction with these pathways.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are anti-microbial agents produced by host cell phagocytes to help control infection. Therefore, the evasion or suppression of the toxic effects of oxidative stress is crucial to the survival of pathogenic bacteria. *C. burnetii,* when phagocytosed by human neutrophils, prevents ROS production by preventing assembly of NADPH oxidase on the phagosomal membrane [56]. *C. burnetii* has also been shown to secret an acid phosphatase (CBU_0335) that inhibits the release of reactive oxygen intermediates [57]. Expression of a subset of DNA repair genes was reported to be strongly upregulated in response to oxidative stress [58]. Interestingly, *C. burnetii* remains microaerophilic, though it has evolved complex mechanisms to evade or resist oxidative stress [59]. Further investigation into this intricate relationship will aid in our understanding of important mechanisms of immune evasion and bacterial persistence.

Secretion systems

Gram-negative bacteria employ several secretion systems to translocate proteins across two lipid bilayers (the inner and outer bacterial membranes) and the host cell membrane. Intracellular bacteria depend on these secretion systems to modulate interactions with the host and subvert host pathways in order to survive. Sec-dependent secretion is a very well-studied, general secretion pathway universal to eubacteria. Most of the Sec system components are found in the *C. burnetii* genome [60]. Other secretion systems predicted to be functional in *C. burnetii* include: a type I secretion system (T1SS); a type IV pilus (T4P)-related type two secretion system (T2SS); and a type IVB secretion system (T4BSS) called Dot/Icm (Defect in organelle trafficking/Intracellular multiplication) [1]. Current understanding of these secretion systems is based largely on homology to *Legionella pneumophila.* The *C. burnetii* genome encodes genes, such as *tolC, enhC,* and *dot/icm*, that are predicted to play roles in the T1S, T4P, and T4B systems, respectively [61].

The canonical Sec pathway functions to transport proteins across the bacterial inner membrane into the periplasm. A recent study suggests outer membrane vesicles (OMVs) contribute to Sec-mediated secretion by *C. burnetii* (Figure 2) [62]. Portions of the outer membrane pinch off from the cell envelope and form OMVs containing periplasmic components, including virulence factors, used to manipulate the environment. Stead et al. recorded obvious membrane blebbing and OMV production during *C. burnetii* growth. The authors hypothesize that OMVs provide a protective environment for secreted cargo inside the harsh parasitophorous vacuole.

Very little is known about the role a T1SS may play in *C. burnetii* infection, but the presence of a *tolC* gene in its genome suggests the organism is competent for type I secretion. Proteins secreted through the T2SS are initially transported into the periplasm through the Sec or Tat system. While *C. burnetii* doesn't have all the components required for T2S, it does encode genes involved in T4P assembly. The type II secretion apparatus in most Gram-negative bacteria is composed of 12-15 proteins. *Francisella* spp. requires fewer proteins to produce functional type 4 pili capable of secretion [63], most of which have homologues in *C. burnetii*. Mutations in the genes involved in these secretion systems would greatly aid in increasing our understanding of the roles they play in *C. burnetii* virulence and survival.

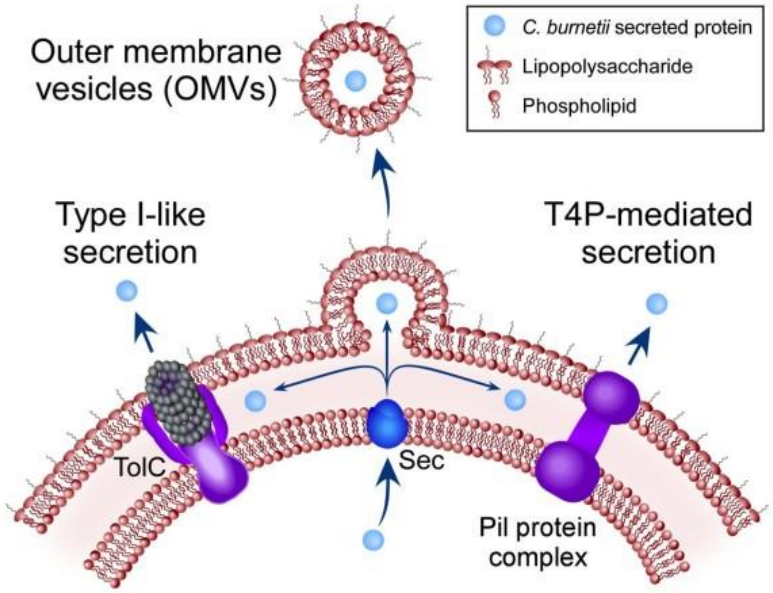

Figure 2: Possible Sec-mediated secretion mechanisms of *C. burnetii*. Proteins transported across the inner membrane by the Sec translocase could be secreted by a T1SS mediated by TolC, a T4P-like T2SS mediated by 13 *pil* genes, or sequestration by OMVs [62].

Many pathogens depend on T4SSs to transport DNA or proteins during infection of a host. The T4SSs are classified into two subgroups, type IVA and type IVB (T4BSS). A functional T4BSS was first discovered to be required for *L. pneumophila* infection by both Ralph Isberg's and Howard Shuman's laboratories [64]. These groups independently named the genes involved in this secretion system as: *dot* (for defect in organelle trafficking) or *icm* (for intracellular multiplication). *C. burnetii* encodes *dot/icm* genes closely related to those found in *L. pneumophila* (Figure 3), some of which have been successfully substituted and function for intracellular replication [65].

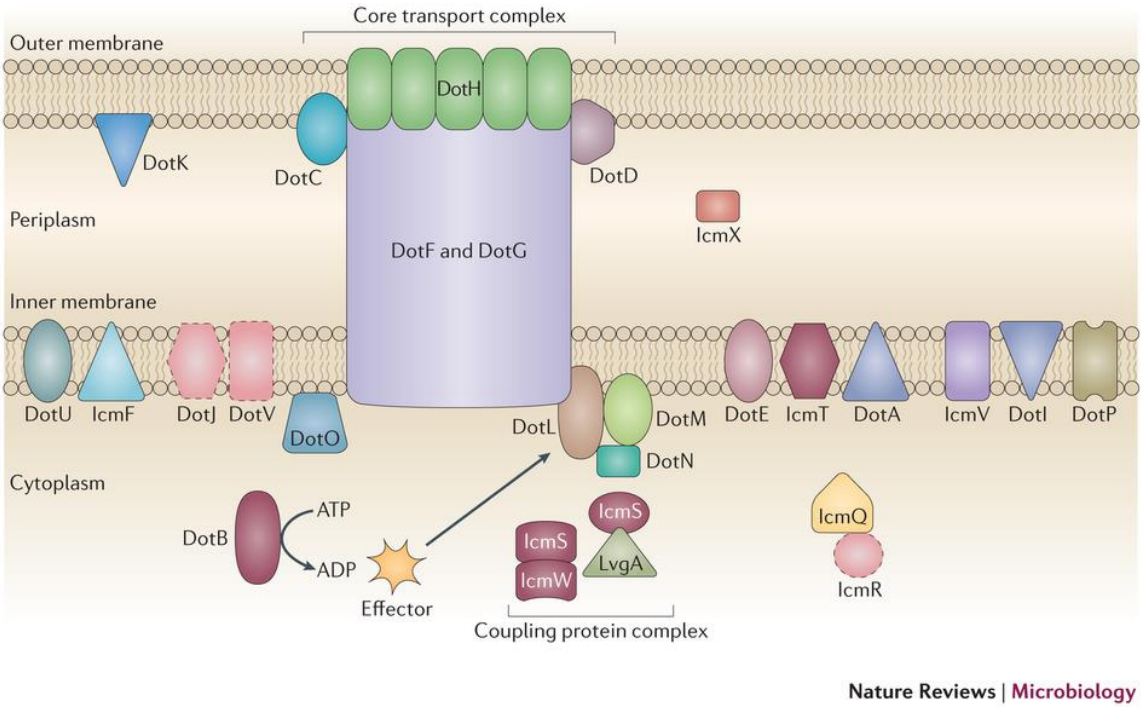

Figure 3: Type IVB secretion system of *C. burnetii*. *Coxiella* encodes 24 T4BSS components, including those that are hypothesized to make up the core transport complex [1].

Before *C. burnetii* was able to be grown outside of host cells, *L. pneumophila* was used as a surrogate host to screen a large collection of candidate protein substrates for the *C. burnetii* Dot/Icm T4BSS. Over 100 proteins have been identified as Dot/Icm substrates from these screens [32,47,66,67].

While the Dot/Icm system functions similarly in *C. burnetii and L. pneumophila,* not all genes correspond directly. Four *Coxiella* genes: *icmS, icmT, icmW,* and *dotB,* restored intracellular growth, either fully or partially, when expressed in a corresponding *L. pneumophila* deletion strain. No complementation was observed, however, with four other *C. burnetii* Dot/Icm homologous genes: *icmB, icmJ, icmO,* and *icmP* [68]. Unlike *L. pneumophila,* which requires effectors to be translocated by the Dot/Icm system within minutes of uptake, *C. burnetii* has been shown to remain viable within the lysosome independent of a functional T4BSS [69]. It is not surprising that the effector proteins would function differently in the two pathogens because *C. burnetii* does not escape the phagosome as other pathogens do. Instead, the Dot/Icm system is necessary to make the environment of the phagolysosome permissive for *C. burnetii* replication [32].

There are still many unanswered questions regarding the structure and function of the secretion systems in *C. burnetii.* The ability to apply genetic tools, including transposon mutant libraries, will undoubtedly increase our understanding of the pathogenic process and lead to the development of more efficient therapies and vaccines.

Animal Model of Disease

Animal models commonly used in the study of Q fever include mice, guinea pigs, nonhuman primates, and livestock. Nonhuman primate models of Q fever are

essential for evaluating vaccine candidates. Rhesus and cynomolgus macaques have been used to assess *C. burnetii* infection since the 1970s [70]. These primates are an excellent model, showing strong similarity to acute disease in humans, including pulmonary radiologic changes, bacteremia, antibody response, and clinical symptoms [71]. However, non-human primate models are expensive, cannot be used in large numbers, and ethically cannot be justified during all stages of the research process. Because of this, small animal models of *C. burnetii* infection are also quite necessary.

The guinea pig model of clinical disease is more relevant for testing vaccines or antibiotic regimens than the mouse model. With this model, fever is the primary indicator of disease, which closely mimics human acute Q fever [70]. The guinea pig aerosol challenge model requires a low dose of *C. burnetii* organisms for the animals to display relevant clinical and pathological evidence of the disease, making this the small animal model of choice for acute Q fever [72]. Guinea pigs have also been used to model Q fever endocarditis by damaging the animals' heart valves using various methods, such as electrocoagulation, prior to exposure with *C. burnetii* [73].

Mice are the most commonly used animal model of Q fever because of the many relevant genetic and immunologic tools available. For example, overexpressing IL-10 in transgenic mice elicits an antibody-mediated (Th2) immune response, which can be used to model chronic Q fever. This model allows for disease pathology to be studied [74]. BALB/c mice are widely used in *C. burnetii* research, including studies to test proteins for protective immunity against challenge with NMI [75]. Though A/J mice are more susceptible to *C. burnetii* infection than other inbred mouse strains [76], they are not as

widely used as the immunocompetent BALB/c or the immunocompromised SCID mice (lacking functional B and T cells). Mice do not present with many clinical signs of illness, so splenomegaly is primarily used for disease determination [77].

In an effort to more closely mimic natural infection and determine the mechanism of pulmonary immunity against *C. burnetii* infection, studies are conducted using aerosol challenge. Although this is a more natural route of infection, it is difficult to precisely determine the number of organisms the animals receive. One study infected BALB/c and SCID mice with 10^8 organisms of either the NMI or Q212 strain (human chronic endocarditis isolate, G). Infection developed in both immunocompetent and immunocompromised mice. [78]. BALB/c and SCID mice were also used to study innate response in lung neutrophils and macrophages after nose-only aerosol challenge with 10^9 bacteria (mice received approximately 10^7 bacteria). This was determined to be a working infection model resulting in splenomegaly and increased genomic copy number in the spleen and lungs [79]. An alternative to aerosol challenge is intratracheal instillation, a technique we have successfully employed in our laboratory. This is more effective than inhalation and results in an exact amount of inoculum delivered deep into the lung [80].

There are several animal models of virulent phase I *C. burnetii* infection available, including guinea pig, mouse and primate models; however, SCID mice are the only animal model that allows high replication of *C. burnetii* NMII infection [81,82]. Acquired immunity is essential for the host to clear infection by PII *C. burnetii.* T cells specifically are necessary for bacterial clearance, while cytokines such as IFN-γ and

TNF-α are important for infection control [15]. Given that most of the recent advances with generating and complementing mutants are conducted with the avirulent NMII strain, a reliable *in vivo* model of infection is needed to characterize the phenotypes associated with these mutations.

Recently, a study of immunocompromised A/J mice was used to evaluate a new treatment to potentially replace doxycycline. A head-only aerosol challenge of A/J mice with was conducted with approximately $6x10^9$ GE/mL of avirulent NMII. The mice received approximately $5x10^6$ bacteria. Liposome-encapsulated ciprofloxacin delivered by the intranasal route was found to improve efficacy and was more efficient than treatment with doxycycline [83]. In the last few months, an insect model of *C. burnetii* infection was developed that is susceptible to *C. burnetii* NMII. This model was used to characterize T4BSS mutants in an *in vivo* system. This is the first non-mammalian *in vivo* model of *C. burnetii* infection, potentially suitable for rapidly characterizing mutant phenotypes and screening of novel antimicrobials [84].

Shortfalls and Recent Advances

Axenic Growth

Until a recent breakthrough in understanding the organism's metabolic pathway, attempts to grow *C. burnetii* outside of the host cell were unsuccessful. The bacterium had little to no metabolic activity in neutral pH buffers, but acid activation buffers (pH 4.5) significantly enhanced *C. burnetii* metabolic potential *in vitro* [85]. This is consistent with the understanding that *C. burnetii* replicates and grows in a lysosome-like parasitophorous vacuole (PV) that maintains a pH of approximately 4.5-5.3 [86].

Unfortunately almost three decades passed before *C. burnetii* was discovered to encode terminal oxidases associated with aerobic and microaerobic respiration. Growth was tested under various oxygen tensions, and it was found that the number of substrates oxidized by *C. burnetii* increases as less oxygen is available [59]. Once *C. burnetii* was defined as a microaerophile, a medium was successfully developed to supported axenic growth of the infectious organism. Axenic growth medium, Acidified Citrate Cysteine Medium (ACCM), using microaerophilic conditions, allows the bacterium to replicate outside of host cells and to replicate as isolated colonies on solid media [59]. Because of this fundamental advance, an increasing number of genetic tools have been adapted, including transposon (Tn) systems to generate random mutants, to *C. burnetii.*

In addition to liquid culture, *C. burnetii* can also be grown as colonies in ACCM-agarose using a soft agarose overlay method [59]. *C. burnetii* colonies are grown in the top medium layer by mixing 10 ml of filter-sterilized 2x ACCM-2 with 10 ml of 1% (wt/vol in water) melted Ultra-Pure agarose (Invitrogen) to create a 0.5% ACCM-2 agarose base in 100- by 20-mm petri dishes. The bacterial inoculum is mixed with 2.5 ml of 0.25% melted ACCM-2 agarose equilibrated to 37 °C, which was prepared by mixing together 1.25 ml of 2x ACCM-2 with 1.25 ml of 0.5% melted agarose. This solution is then poured on top of the solidified ACCM-2 agarose base. Plates are incubated for 7-10 days as described above for ACCM-2 to allow colony development. After approximately 7-10 days incubation, small colonies become visible by the naked eye [59] . Colonies can be picked from the agarose by lightly touching them with a micro-pipette tip, then expanded in ACCM-2 liquid culture.

Genetic Tools

Transformations

Transforming any obligate intracellular bacterium has technical constraints associated with it because the pathogen relies on the host cell for replication [87]. *Coxiella* must be purified from host cells before genetic transformation. Because of its biphasic lifestyle, bacteria should be purified when host cells contain roughly equal numbers of LCVs and SCVs [88]. The first successful transformation of a stably maintained exogenous plasmid into *C. burnetii* was reported nearly twenty years ago[89]. This came just two years after the first obligate intracellular bacterium (*Chlamydia trachomatis)* was transformed successfully, proving electroporation could be used to genetically transform an obligate intracellular bacterium [90]. Integration occurred by homologous recombination between the plasmid autonomous replication sequence (ars) [91] and the *C. burnetii* genome. This was followed by a very lengthy selection and expansion process, taking 2-3 months for ampicillin-resistant *C. burnetii* to be recovered from host cells [89]. However, ampicillin-resistant organisms were discovered without the β-lactamase gene, indicating the long selection process allowed spontaneous mutation [89]. Still, this was a great advance in *Coxiella* genetics, demonstrating electroporation could be used to introduce DNA into *C. burnetii.*

Transposon mutagenesis

Transposons (Tn) are ‘jumping genes’ that can change their position in the genome. These genes can be isolated and put on a plasmid for integration into target cell genomes [92]. A transposon that inserts into a functional gene will produce a mutation

that will likely disrupt gene function; therefore, transposons have been used in a wide variety of mutagenesis studies [92]. *Mariner* or *mariner*-like elements are a diverse family of transposons from insects, nematods, flatworms, and humans [93]. Lampe *et al.* purified the *Himar1* transposase from the horn fly, *Haematobia irritans,* and discovered that it uses a cut-and-paste transposition pathway that inserts exclusively into a TA dinucleotide [93]. Recently, genetic transformation was used to successfully generate a defined gene mutation in *C. burnetii* using the *mariner*-based *Himar1* transposon system [88]. In this system, transformation with *Himar1* required two different suicide plasmids, encoding either the transposon or transposase, and containing a ColE1 origin of replication for rescue cloning, chloramphenicol acetyltransferase (cat^R, for chloramphenicol resistance), and mCherry red fluorescent protein genes [88]. Vero cells were infected with *C. burnetii* organisms transformed with the two-plasmid *Himar1* system, which resulted in 35 unique Tn insertion sites. This is a very time intensive process using host cells, and clonal isolation and expansion of transformants can take 8-12 weeks [88]. Development of ACCM growth medium and semi-solid ACCM agarose dramatically reduces time to expand transformants to only 16 days. Importantly, this also allows recovery of transformants that are not capable of growth *in vitro* [94].

Himar1-based mutant libraries generally result in robust genome coverage due to high transposition frequency and low target site specificity (TA dinucleotide). Members of our laboratory refined the two-plasmid system described by Beare, *et al.* by establishing a stable single plasmid system, designated pKM225, replacing promoter regions of all critical selection markers with *C. burnetii*-specific promoter regions [95]

(Figure 4). The development of a clonal Tn mutant library will significantly enhance characterization of *C. burnetii* secretions systems and increase our understanding of specific mechanisms of its virulence.

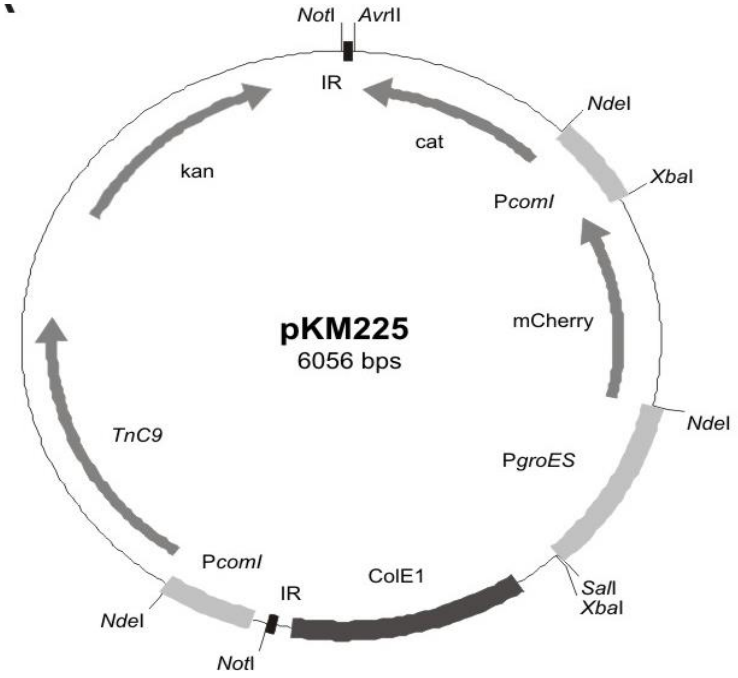

Figure 4: Map of pKM225 plasmid for single-plasmid delivery of *Himar1* transposon system. The ColE1 origin of replication, mCherry fluorescence marker, and chloramphenicol resistance gene (cat) are contained within the inverted repeats (IR) and are inserted into the genome upon transformation of the plasmid. The transposable element, TnC9, is outside the IRs and is therefore not inserted, which keeps the plasmid from 'jumping' out of a gene once it is inserted.

Complementation

Transposon mutagenesis is a powerful tool to identify bacterial genes important for pathogenesis and subversion of the host immune system. To rule out the possibility that observed mutant phenotypes could be due to unlinked loss-of-function mutations, it

is important to validate these phenotypes with complementation studies [53]. Chloramphenicol and kanamycin have both been established as selectable markers in *C. burnetii* [96]. This allows a Tn mutant expressing one marker to be complemented with a *Himar1* construct expressing the other marker. Beare, *et al.* successfully complemented an *icmD* mutant using a Tn7-based transposon system to introduce the *icmDJB* operon into the chromosome in the *glmS*-CBU_1788 intergenic region [69]. They used the same method to complement *dotB* and *dotA* mutants a year later [97]. This complementation strategy permits verification of virulence factors discovered by transposon mutagenesis.

Conclusion

Q fever, a zoonosis caused by the Gram-negative bacterium *C. burnetii,* has been described in nearly every country worldwide. Q fever is not only an occupational hazard for livestock workers, but also a potential biological weapon. The organism is extremely stable, efficiently aerosolized and disseminated, and can remain viable over long periods. This coupled with its low infectious dose has led the CDC to classify *C. burnetii* as a Category B Select Agent [98]. Though *C. burnetii* was first isolated nearly 80 years ago, its mechanisms of disease are poorly understood. This is largely due to its intracellular lifestyle; however, recent advances have significantly progressed *Coxiella* research in the last few years. First, there was the development of a liquid medium allowing growth outside of the host cell, followed by colony isolation on solid medium, a transposon system to generate mutant libraries, and complementation studies to verify phenotypes. The overall goal of these experiments was to generate transposon mutant libraries in *C. burnetii* and then use these libraries to identify genes essential for growth

in vitro and *in vivo*. We optimized methods to generate defined transposon mutants, resulting in mutations in nearly 20% of the predicted ORFs. Included in these mutants were a number of bioinformatically predicted virulence factors that we further studied in cellular and animal models of infection.

CHAPTER II

COXIELLA BURNETII MUTANT LIBRARIES

All procedures and strains used in this work were approved by the Texas A&M University Office of Biosafety (IBC permit #2012080) and the Institutional Animal Care and Use Committee (AUP #2013-0138).

Background

Members of our laboratory established a stable single plasmid transposon system, designated pKM225, with *C. burnetii*-specific promoter regions for all critical selection markers [95]. This system was used to develop a clonal Tn mutant library, which will significantly enhance characterization of *C. burnetii* secretions systems and increase our understanding of specific mechanisms of its virulence. Colony growth was used for selection and isolation of *C. burnetii* insertion mutants. *C. burnetii* was transformed with pKM225 and grown in the presence of 5 μl/ml chloramphenicol (Cm). Individual mutant clones were propagated on agar plates, cultivated, stored and characterized genetically for location of insertion by rescue cloning (Figure 5). Transposon insertions were homogeneously distributed throughout the chromosome and plasmid; however, we also encountered several "hot spots" where the transposon inserted for multiple clones.

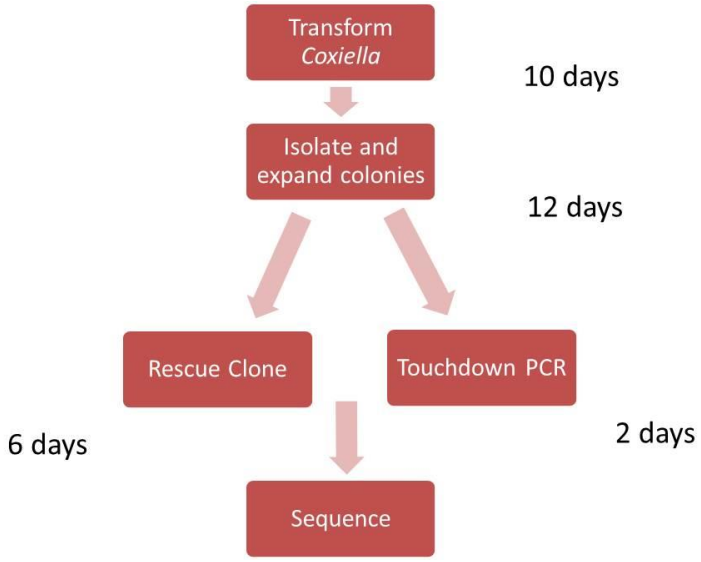

Figure 5: Methods for isolation and insert characterization of each *C. burnetii* mutant.

There are roughly 120,000 TA sites evenly distributed in the *C. burnetii* genome among nearly 2100 ORFs [38]. We hypothesize a mutant library of approximately 25,000 independent clones would saturate the genome. Most or all of the nonessential genes would contain insertions, and a subset of lethal gene disruptions would identify essential genes in the *C. burnetii* genome. Pooled libraries were generated by transforming *C. burnetii* with pKM225 using conditions described for the clonal library. Cells were recovered in 6 mL ACCM-2 for 4-5 days before being expanded to 40 mL and cultured for another 4 days. Pooled transformations were either analyzed individually or combined into a genome saturation pool (Figure 6). Estimating 500-1000 inserts per transformation based on colony counts from plated transformations in the defined mutant library, 35 transformations should generate the 25,000 clones needed to saturate the genome.

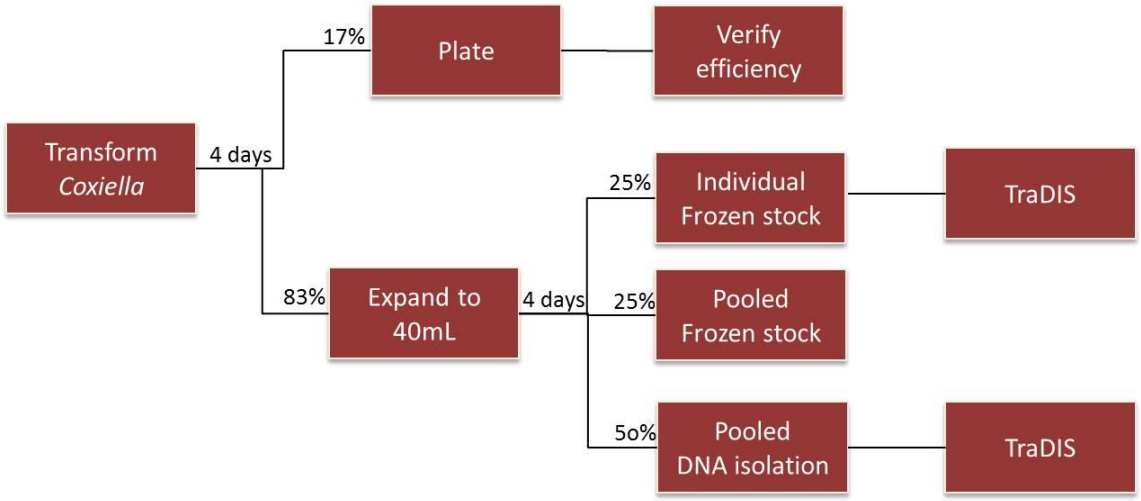

Figure 6: Methods for generating pools of transposon mutants to saturate the *C. burnetii* genome.

Mutant libraries were analyzed by one of two transposon sequencing approaches: high-throughput insertion tracking by deep sequencing (HITS) [99] or transposon-directed insertion-site sequencing (TraDIS) [100]. These techniques for high-throughput sequencing of transposon-insertion sites were developed concurrently and follow the same basic workflow (Figure 7): transposon mutagenesis and construction of pools of single insertion mutants; enrichment of transposon-insertion junctions, purification and PCR, and, finally, sequencing. Another method, transposon sequencing (Tn-seq) [101], uses enzyme digest instead of manual shearing to cut the bacterial genome while leaving the transposon insert intact.

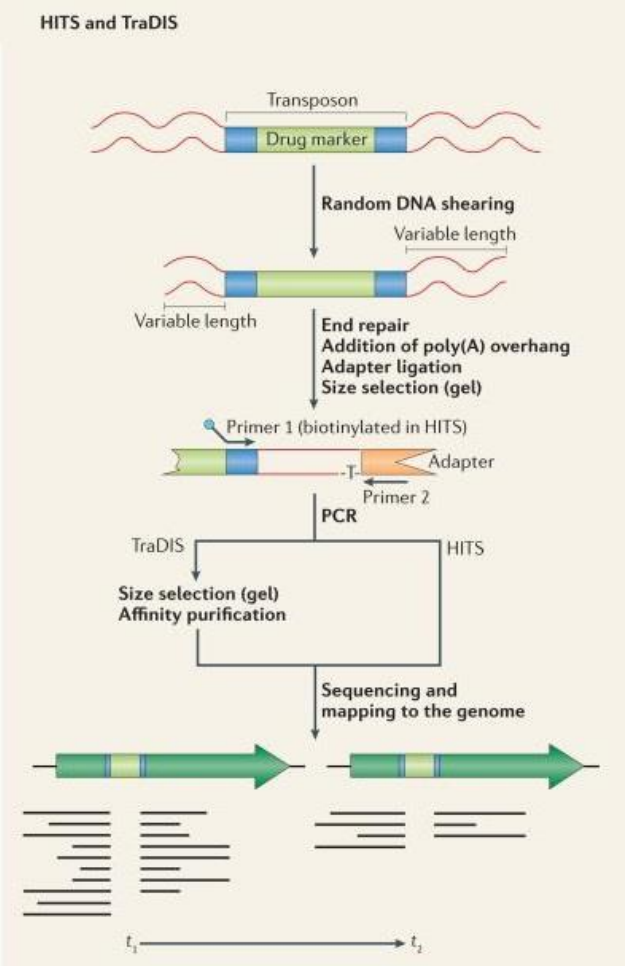

Figure 7: Transposon insertion sequencing methods. (modified from van Opijnen and Camilli [83]) Two methods of transposon sequencing are illustrated. These methods are very similar, beginning with a pool of genomic DNA from the Tn insertion library and ending with sequencing of the Tn junctions.

The aim of this study was to generate a large and diverse *C. burnetii* transposon mutant library near saturation for all non-essential ORFs in ACCM in order to map *C. burnetii* genes essential for growth and viability. We also expect to identify a set of basic structure and metabolic related genes that are required for survival and replication in ACCM. We expect that 200-300 ORFs are essential for *C. burnetii* growth in ACCM.

ORFs not represented by insertions in the pooled library should also be unavailable in the defined insertion library. Currently, only two virulence factors have been confirmed for *C. burnetii:* LPS and a type IVb secretion system [34,35]. The identification of additional *C. burnetii* virulence factors using recently developed genetic techniques will be instrumental in finding targets for new therapies.

Materials and Methods

Bacterial Strains, cell lines, and growth conditions

Escherichia coli strain DH5α was propagated at 37 °C in Luria-Bertani (LB) broth or agar, purchased from Difco (Sparks, MD). Media were autoclaved to 121 °C for 30 minutes and supplemented with the appropriate filter sterilized antibiotic(s) at the final concentrations listed: chloramphenicol (Cm) (34 μg/ml) or kanamycin (Kan) (50 μg/ml). Broth cultures were usually grown overnight (O.N.) in 5 ml of LB and shaken at 200 rpm. Selected strains were stored at -80 °C after resuspending 5 ml of O.N. culture in 1 ml of autoclaved glycerol (10% v/v) in LB without antibiotics.

The laboratory derived *C. burnetii* Nine Mile phase II (NMII), strain RSA 439 clone 4, was cultivated in T-75 cell culture flasks or 0.2-μm-pore-size-filter-capped 125-ml Erlenmeyer flasks containing 75 ml of medium or T-25 flasks containing 20 ml of medium grown in filter sterilized liquid ACCM-2 or ACCM-agarose. Cultures were grown for approximately 7 days at 37 °C in a 2.5% O_2 and 5% CO_2 environment. Oxygen was displaced by nitrogen gas. Where required, Cm was used at 5 μg/ml.

Transposon mutagenesis of C. burnetii NMII

We used pKM225, a *Himar1*-based single plasmid transposon system, to inactivate a large fraction of the genome. *C. burnetii*, axenically cultured to stationary phase in ACCM-2, was washed twice and resuspended in water to an approximate concentration of 1 X 10^9. 1µg of pKM225 plasmid DNA was added to 50 µl bacteria and electroporated under the following conditions: 2.5kV, 200Ω, 25µF. Following electroporation, the bacteria were recovered O.N. in 6 mL ACCM-2. The following day, 5 µg/mL Cm was added to bacterial cultures. Bacteria was then grown for an additional 2-3 days and plated on ACCM-agarose plates containing chloramphenicol.

Colony Formation

C. burnetii colonies were established using a modified soft agarose overlay method in which bacteria are grown in the top medium layer. A 0.5% ACCM-2 agarose base in 100- by 20-mm petri dishes was created by mixing 10 ml of filter-sterilized 2x ACCM-2 with 10 ml of 1% (wt/vol in water) melted Ultra-Pure agarose (Invitrogen). The bacterial inoculum was mixed with 2.5 ml of 0.25% melted ACCM-2 agarose equilibrated to 37 °C, which was prepared by mixing together 1.25 ml of 2x ACCM-2 with 1.25 ml of 0.5% melted agarose. This solution was poured on top of the solidified ACCM-2 agarose base. Plates were refrigerated (4 °C) for 30 min to aid solidification of the top agarose and then placed in a laminar airflow biosafety cabinet with lids ajar for 20 min to remove condensation. Plates were incubated for 7-10 days as described above for ACCM-2 to allow colony development. After approximately 7-10 days incubation, small colonies become visible by the naked eye. Single colonies were isolated and

resuspended in 500ul ACCM-2 in 48 well plates. Following an additional 4-5 days, cultures were expanded to 20mL for another 7 days. We then pelleted the cultures and isolated DNA for rescue cloning.

DNA isolation and rescue cloning

After approximately 7 days, the turbid bacterial culture was transferred to a 40 ml oakridge tube and spun at 15k rpm for 20 minutes at 4 °C. The pellet was resuspended and transferred to a 1.5 ml eppendorph microcentrifuge tube and spun again at 15k rpm for 20 minutes at 4 °C. Genomic DNA was isolated for rescue cloning of Himar1-containing fragments following the manufacturer's directions (GenElute Bacterial Genomic DNA Kit, Sigma-Aldrich, St. Louis, MO), eluted in 50 µl of elution solution. Purified DNA was digested with the HindIII high fidelity (HF) enzyme (NEB) by adding 2 µl enzyme and 5.2 µl cutsmart buffer and incubating the reaction at 37 °C for 2h. The enzyme was then heat inactivated at 80 °C for 20 minutes. Ligation reactions were carried out in a 16 °C water bath O.N. by adding 5.7 µl of 10X T4 DNA ligase buffer and 2 µl of T4 DNA ligase to the reaction tube.

Transformation

To prepare *E. coli* chemically competent cells, a single colony was inoculated in 5 ml of LB and incubated O.N. at 37 °C while shaking at 200 rpm. Two ml of the O.N. culture was used to inoculate 200 ml of LB medium in a 500 ml flask. Once the OD_{600} reached 0.6-0.8, the culture was chilled on ice for 15 minutes. The cells were centrifuged at 3300g for 10 minutes at 4 °C. The pellet was resuspended by gently swirling 30-40 ml of cold 0.1 M $CaCl_2$ and incubated on ice for 30 minutes. The cells were centrifuged

again at 3300g for 10 minutes at 4 °C and gently resuspended in 2 ml of cold 0.1 M $CaCl_2$ plus glycerol (15% w/v). Five hundred µl of competent cells were transferred to microcentrifuge tubes and frozen at -80 °C. Chemical transformation was performed by adding 50 µl gently thawed competent cells to the entire ligation reaction. After gentle mixing, the sample was incubated on ice for 30 minutes. Heat shock was performed at 42 °C for 45 seconds and the reaction was immediately returned to ice. One hundred µl of SOC media (900 ml dH_2O, 20 g bacto tryptone, 5 g bacto yeast, 2 ml 5 M NaCl, 2.5 ml 1 M KCl, 10 ml 1 M $MgCl_2$, 10 ml 1 M $MgSO_4$, 20 ml of 1 M glucose) was added to the sample and allowed to recover at 37 °C for 1 hour prior to plating on selective media. Plates were incubated at 37 °C for 2 days, when pink colonies became visible. One to three colonies per transformation were picked and inoculated in 5 ml of LB with Cm and incubated O.N. at 37 °C while shaking at 200 rpm.

Plasmid isolation

Plasmids were isolated from bacterial O.N. cultures by alkaline-SDS lysis following the manufacturer's directions (Thermo GeneJet Plasmid Miniprep Kit). Plasmid concentrations and purity were determined by evaluating the $OD_{260/280}$ and $OD_{260/230}$ values determined from a NanoDrop Spectrophotometer. An $OD_{260/280}$ value greater than 1.8 and $OD_{260/230}$ greater than 2.0 indicated the sample was pure enough for sequencing and reliable genetic manipulation.

Touchdown PCR

As an alternative to rescue cloning and transformation into *E. coli*, we used touchdown PCR to identify Tn insertions in the genome [102]. PCR reactions were

performed in a 50 µl mixture containing 100 pmol hybrid primer (HIB17), 20 pmol Tn specific primer (ColE1-R), 100-400 ng gDNA template, 1x PCR buffer (60 mM Tris-SO_4, 18 mM NH_4SO_4), 0.2 mM each dNTP, and 1.5 U of Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen). PCR consisted of two phases: phase 1 included an initial step of 95 ℃ for 5 min, followed by 25 cycles of denaturation at 95 ℃ for 45s, annealing at variable temperatures for 45 s, and extension at 72 ℃ for 2 min. In the first cycle, the annealing temperature was set to 60 ℃ and, at each of the 24 subsequent cycles, the annealing temperature was decreased by 0.5 ℃ per cycle down to 47.5 ℃. Phase 2 consisted of 25 cycles of 95 ℃ for 45 s, 50 ℃ for 45 s, and 72 ℃ for 2 min. After the last PCR cycle, the samples were cooled to 4 ℃, and a 6 µl aliquot of the amplification products was electrophoresed on a 1% agarose gel (1x TAE), stained with ethidium bromide, and visualized under ultraviolet (UV) light.

Sequencing plasmids for transposon insertions

Plasmids or PCR products were sent to Europhins MWG Operon (Huntsville, AL) for sequencing in a 96 well plate with 8 µl plasmid DNA and 4 µl primer (CatF or ColE1-R) per well to determine genomic insertion site of Himar1 transposon. Sequences of primers obtained from Integrated DNA Technologies (San Diego, CA) are listed in Appendix C.

Analysis of Transposon-Insertion sites

For mapping insertion position within the genome, the sequence reads obtained were aligned (BlastN) against the *C. burnetii* phase I (RSA 493) whole genome sequence [60]. For each analyzed sequence, the inverted terminal repeat (ITR) region,

5’-ACAGGTTGGCTGATAAGTCCCCGGTCTC-3’, which flanks the transposon, was localized and the location where the transposon was inserted within the genome was determined by identifying the start and end positions of the matching sequence with respect to the genome sequence.

Generation of Pooled Libraries

Approximately 500 independent mutants were expected to be generated per transformation. In order to obtain saturation of the *C. burnetii* genome, 50 independent transformations were performed with pKM225 as described above, except transformations were not plated on ACCM-agarose. Instead, each transformation was recovered in ACCM-2 for 5-7 days then stored separately as well as pooled into a high-density transposon mutant library. *C. burnetii* transposon pooled libraries were grown and DNA isolated as described above for individual cultures.

Preparation of pooled libraries for sequencing: HITS

Analysis of the input libraries will be performed to demonstrate uniform distribution in chromosomes and the identification of genes essential for survival in ACCM using HITS. Genomic DNA from pooled *C. burnetii* Tn libraries was diluted to 1 µg in 130 µl total volume in TE buffer. DNA was sheared to 300 bp on a Covaris S220 ultrasonicator using the following settings: duty cycle = 10%, intensity = 4, 200 cycles / burst, time = 27 s. Size selection with AMPure XP beads (Beckman Coulter) following manufacturer’s instructions. (Always mix 1x beads). Next, Quick Blunting end repair was performed at 25 °C for 30 min. PCR purify and elute in 35 µl. A 50 µl total A-tailing reaction was set up using 5 µl NEB buffer 2, 10 µl 250 µM dATP, and 2 µl

Klenow 3'-5' exo (5 U/ml), and incubated at 37 °C for 30 min. PCR purify and elute in 45 µl. Adaptors were ligated by adding 1 µl Promega T4 DNA Ligase (3 U/µl), 1 µl Adaptor B (3' adaptor ligates both ends, T-overhang), and 5 µl buffer, and incubating 4 hours at 15 °C followed by 15 min at 65 °C to inactivate the enzyme. The reaction was bead purified twice to remove all extra adaptors and eluted in 40 µl. The first PCR reaction, to add the 5' adaptor (partial) to the Tn sequence, was carried out in a 100 µl total volume using 10 ng DNA, Phusion HF buffer, dNTPs, Himar Primer with MID sequence, PCR 2.0 primer, and Phusion DNA polymerase (2 U/µl) under the following conditions: 98 °C for 10 s, followed by 12 cycles of 98 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and a final extension of 72 °C for 10 min. Bead purify and elute in 40 µl. The second PCR reaction, to pre-select, add biotin and the rest of the 5' adaptor, was carried out in a 200 µl total volume using 125 ng DNA, 5x Phusion HF buffer, 10 µM Preselect F1 primer, specific INDX primer, dNTPs, and Phusion DNA polymerase under the same conditions as the first PCR, except with 20 cycles instead of 12. PCR purify and elute in 55 µl. One to 2 µg DNA was added to DynaBeads (Dynal streptavidin-coated beads, Invitrogen) to capture and wash the sample, followed by heat denaturation and DNA isolation. The final PCR, used to amplify the Tn-insertion product was conducted in 100 µl total volume using 5 ng DNA, 5x Phusion HF buffer, 10 µM PCR 1.0 primer, 1 µM PCR 2.0 primer, 10 µM INDX primer, dNTPs, and Phusion *taq* DNA polymerase under the following conditions: 98 °C for 30 s, followed by 8 cycles of 98 °C for 10 s, 58 °C for 30 s, and 72 °C for 20 s, with a final extension of 72 °C for 5 min. Bead purify and elute in 35 µl. The final product was then analyzed for fragment length

on the Bioanalyzer using the High Sensitivity DNA Assay kit following the manufacturer's directions.

Preparation of pooled libraries for sequencing: TraDIS

Libraries for sequencing were prepared from 2.5- 5 µg of genomic DNA extracted from transposon libraries. Lo-bind Eppendorf tubes were used throughout the protocol. DNA was fragmented using a Bioruptor sonicator for 30 min with 30 s on/off pulses with medium intensity. Fragmented DNA was purified using the Qiaquick PCR Purification kit (Qiagen) according to the manufacturer's instructions. DNA was eluted in 40 µl EB buffer, followed by 40 µl pure water and eluates were pooled. 1 µl of the purified sample was analyzed on a BioAnalyzer (Agilent) using a DNA7500 chip. The mean size of the DNA fragments should be below 500 bp. Next, fragments <150-bp in size, which would be too small for sequencing, were eliminated from the samples using the GeneRead size selection kit (Qiagen) according to the manufacturer's instructions. DNA was eluted in 3x 25 µl EB buffer. End repair and dA-tailing of end-repaired DNA was performed using the NEBNext DNA library prep reagent set for Illumina (NewEngland Biolabs) according to the manufacturer's instructions, with a DNA clean-up after both steps using the Qiaquick PCR Purification kit (35 µl elution volume) and the MinElute PCR purification kit (Qiagen; 22 µl elution volume), respectively. 1 µl of the sample was analyzed on a BioAnalyzer in order to obtain values for the concentration of sample and mean fragment size. Adapter ligation was performed using the following adapters: Adapter-1 (5'-GATCGGAAGAGCACACGTC*T) and Adapter PCR-1 (5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T), where *

symbolizes a phosphorothioate modification, which had previously been phosphorylated and annealed by incubating 20 µl of each oligonucleotide with 5 µl 10x T4 DNA ligase buffer and 5 µl 10U/µl T4 Polynucleotide Kinase enzyme (both NewEngland Biolabs). Annealed adapters were ligated to the A-tailed DNA fragments by mixing 17.5 µl of the DNA sample plus the calculated volume of annealed adapters plus 5 µl Quick T4 DNA Ligase with an equal volume of 2x Quick Ligation Reaction Buffer as part of the NEBNext DNA library prep reagent set. DNA was purified using the Qiaquick PCR Purification kit and eluted in 35 µl PE buffer. PCR enrichment for transposon-containing fragments was carried out on small amounts of the ligated material as parallel PCRs in order to minimize amplification bias. Eight PCR reactions in volumes of 50 µl were set-up per sample consisting of: 41.9 µl nuclease-free water, 5 µl 10x PCR buffer with $MgCl_2$, 1 µl 10 mM dNTP mix, 0.3 µl 100 µM transposon-specific PCR-3 primer, 0.3 µl 100 µM barcoded multiplexing primer (different MPX primer for each library), 1 µl adapter-ligated DNA fragments as template, and 0.5 µl JumpStart Taq DNA polymerase (Sigma Aldrich). The thermal cycling conditions consisted of 10 min initial denaturation at 94°C, followed by 20 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s, followed by a final extension step at 72°C for 10 min. PCR products were pooled and precipitated overnight at -20°C using sodium acetate/ethanol. DNA pellets were resuspended in 75 µl of buffer EB.

1 µl of the sample was analysed on a BioAnalyzer in order to obtain values for the concentration of sample and mean fragment size. The yield of transposon-specific and total PCR products was also determined by qPCR. The concentration of total and Tn-

specific PCR products in each sample was determined from the standard curve and was corrected for the differences in fragments size between the samples (determined using the BioAnalyser) and the standards (=452 bp each). The aim was to obtain samples with >50% Tn-specific PCR products. PCR products were size selected by dry-loading the samples onto a 2% agarose gel in 1x TBE buffer and electrophoresis at 60V for 90 minutes. For sequencing, each library was diluted to 2mM and libraries were pooled using equal volumes.

Preparation of pooled libraries for sequencing: Tn-seq

Genomic DNA from pooled *C. burnetii* Tn libraries was diluted to 1 µg in 130 µl total volume in TE buffer. Purified DNA was digested to approximately 300 bp with the Sau3A1 restriction enzyme (NEB) by adding 2 µl enzyme, 5.5 µl NEB buffer 1, and 5.5 µl BSA and incubating the reaction at 37 °C for 2h. The enzyme was then heat inactivated at 65 °C for 20 min.

Sequencing of pooled libraries

The resulting DNAs prepared by one of the three methods described above will be cluster amplified and sequenced on a HiSeq2500 sequencer (Illumina) as 100 bp single read runs. The Illumina sequencing reads that contain the *Himar1* inverted terminal repeats (ITRs) and the adjacent TA insertion site will be identified and aligned to the *C. burnetii* RSA493 genome sequence. The complexity of the input library will be determined and the number of interrupted ORFs will be analyzed after bioinformatics assembly. Insertions in genes that are significantly under-represented or missing in the

population of mutants will be considered as putative genes that are required for growth or survival in ACCM.

Results

Defined Transposon Mutant Library

A library of *Coxiella burnetii* mutants was generated by transposon mutagenesis. The plasmid pKM225 encoding a *Himar1* transposase was used to introduce a transposon encoding a mCherry fluorescent protein and chloramphenicol resistance randomly onto the genome of the *C. burnetii* NMII strain RSA439. The *C. burnetii* mutants generated were isolated on ACCM-agarose plates in the presence of chloramphenicol and further amplified in liquid ACCM-2 supplemented with chloramphenicol for an additional 12 days.

The mutagenesis procedure was optimized to yield the greatest number of mutated colonies and efficient growth procedures once colonies were isolated, and the rescue cloning efficiency was optimized to greater than 50%. The purified plasmids were sequenced using the transposon-specific primer, CatF, which recognizes a sequence in the 3' region of the Chloramphenicol Acetyltransferase (CAT) gene. The sequences were aligned on the *C. burnetii* RSA493 annotated genome using automated sequence analysis software. This analysis confirmed that isolated clones had single transposon insertions and were distributed homogeneously throughout the genome. Approximately 3000 transposon mutants were isolated as single clones from 30 independent transformations. Of these, approximately 960 were successfully rescue cloned and sequenced to identify the transposon insertion sites (Figure 8). Overall, 800 transposon

insertions were found within *C. burnetii* annotated ORFs and 160 in intergenic regions of the genome (Table 1). The full, detailed list of all transposon insertions identified is included as a separate file.

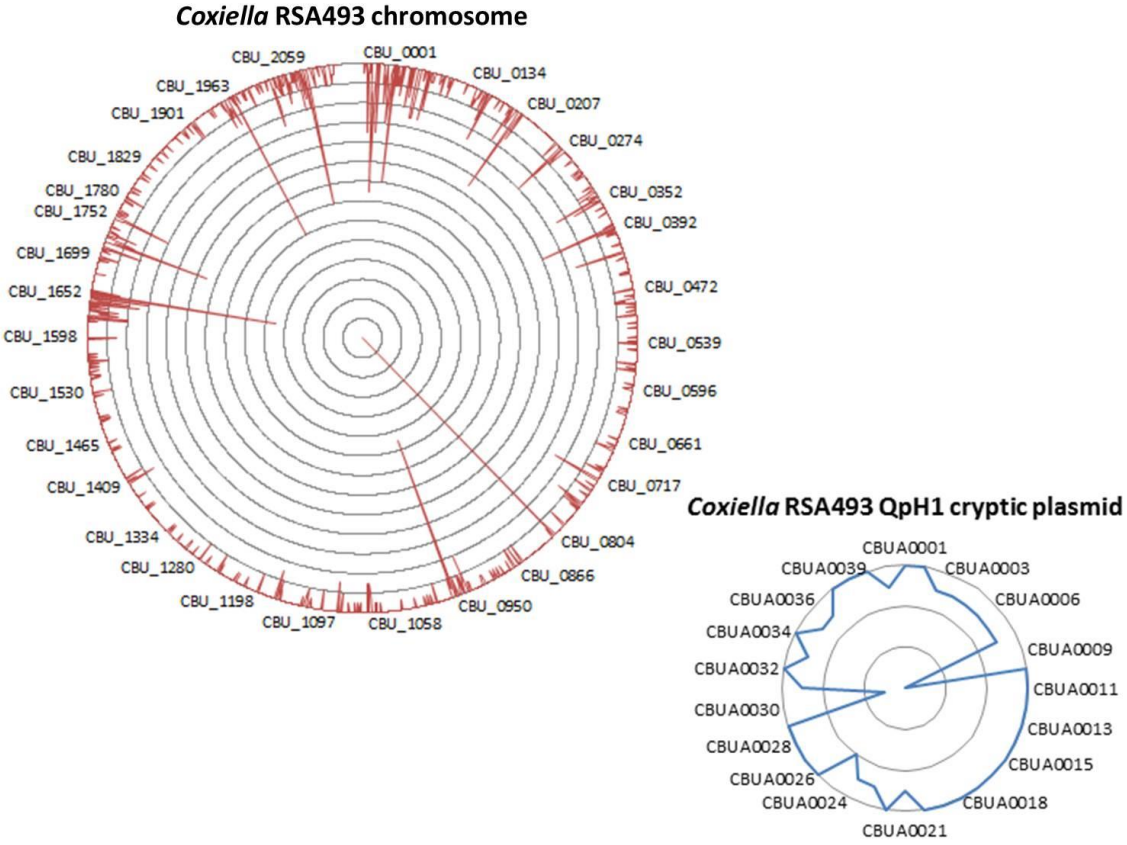

Figure 8: Sequenced transposon insertions. Transposon insertions were annotated on the *C. burnetii* RSA493 chromosome (large circle) and QpH1 cryptic plasmid (small circle). Peaks indicate the site of insertion of each transposon and the height of the peak corresponds to the frequency of mutants isolated presenting a transposon insertion in a given site (1 inner circle corresponds to 2 insertions).

Table 1: Current progress of *C. burnetii* defined transposon mutant library. Nearly 1,000 transformants have been isolated and identified. Less than 10% of these are non-clonal. Approximately 15% of the clonal mutants are intergenic, leaving approximately 800 clonal mutants in predicted ORFs.

Clonal Mutants (Total)	Clonal Mutants (intergenic)	Clonal Mutants (in ORFs)	Clonal Mutants (unique ORFs)	Clonal Mutants (duplicates)	Non-Clonal Mutants
961	160	801	390	290	85

Mutations occurred in 373 ORFs on the *C. burnetii* chromosome and 17 ORFs on the QpH1 plasmid. This corresponds to approximately 18.2% and 37% of the total ORFs present on the chromosome and plasmid, respectively. There were several areas of preferential transposon insertion, which could be due to a high AT content. The genes with the most insertions include: CBU_0804 (RND transporter protein), CBU_0950 (hypothetical protein), CBU_1652 (icmX), CBU_1963 (hypothetical), and CBU_2059 (T4SS substrate, *cirE*). We also generated approximately 60 insertions in *dot/icm* genes. A few of these genes are over 2 kb long, which would allow for more insertion sites.

There were a number of non-mutated regions (Table 2). Some of these gaps were expected since we compared our transposon insertions, which were generated in NMII, to the annotated NMI genome. There is a region of approximately 20 ORFs encoding for LPS (CBU_0679 – CBU_0698) in the NMI genome that is deleted in the NMII genome [38]. There was a large non-mutated region between CBU_0212 and CBU_0264, which encodes ribosomal proteins that are likely essential for bacterial survival in all conditions. We also saw six other regions of about 20 – 30 continuous ORFs each that

did not contain mutations. These could be due to mutations in essential genes or simply areas that we have not yet identified a transposon insert.

Table 2: Non-mutated regions in defined Tn mutant library. These could be due to mutations in essential genes or simply areas that inserts have not yet been isolated.

Non-mutated region	Predicted function
CBU_0115 – CBU_0132	Cell division proteins; ligases
CBU_0212 – CBU_0264	Ribosomal proteins
CBU_0599 – CBU_0622	Outer membrane protein precursors; acyltransferases and other enzymes
CBU_0630 – CBU_0660	Enzymes: synthase, kinase, deaminase, dehydrogenase, and phosphodiesterase
CBU_0677 – CBU_0700	LPS
CBU_0808 – CBU_0854	Transcriptional regulators and activators, biosynthesis proteins, and enzymes
CBU_1335 – CBU_1369	DNA polymerase, cell division proteins, and iron-sulfur cluster assembly proteins
CBU_1417 – CBU_1453	Transcription, translation, and DNA repair proteins; NADH-ubiquinone oxidoreductase chain

Pooled Transposon Mutant Library

High-throughput Insertion Tracking by Deep Sequencing (HITS) and Transposon Directed Insertion Site Sequencing (TraDIS) represent two methods for a high-throughput functional analysis of every *Coxiella burnetii* gene. Using these methods, all the genes required for viability (and therefore all the targets for novel drug design) can be identified in a single experiment [99,100]. These methods have the potential for primarily random insertions of the transposon into the chromosome, that enables equal coverage and that every single gene is targeted in theory. However, insertion of the *Himar1* transposon into the chromosome is not totally random but rather depends on the

presence of TA sites [103]. In order to test the suitability of a *Himar* transposon mutant pool for deep sequencing, the genome of *C. burnetii* RSA493 was searched for TA sites using the Artemis motif finder function [104]. A total of 124,724 TA sites were identified in the ~2Mbp RSA493 genome, resulting in an average distance of 16 bp between sites. No genes without at least one TA site were found, thereby confirming that *Himar1* mutagenesis is suitable for transposon-insertion sequencing in the *C. burnetii* genome.

Initially, we set out to analyze our transposon mutant pools in order to determine redundancy and the number of pools needed to saturate the *C. burnetii* genome with mutations. Transformations yielding 250 independent clones would require 100 transformations to saturate the genome, while a transformation yielding 2500 clones would only require 10 transformations. Based on our experience, the number of clones generated by each transformation is expected to average within this range. *C. burnetii* RSA439 was transformed with 1 μg of the pKM225 plasmid as described above. The transformations were recovered in 20 mL ACCM-2 for 5 days instead of being plated on ACCM-agarose. Five mL of the culture was pelleted and stored at -80 °C, while the remaining 15 mL was pelleted and DNA isolated for analysis by deep sequencing. We compared various purification kits to ensure the greatest yield and purity of genomic DNA from the pooled samples. These samples were delivered to our collaborators at Texas A&M AgriLife Genomics and Bioinformatics Service for library preparation and sequencing on the HiSeq 2500 System (Illumina). Samples were manually sheared on an ultrasonicator (Covaris) to an average of 300 bp, followed by a size selection using

AMPureXP beads (Beckman Coulter). End repair, A-tailing, and adapter ligation were performed with purification after each step. PCR was performed to add the 5' adapter to the transposon sequence. Following a second PCR to preselect and add biotin, the remaining portion of the adapter was added. Products with Tn insertions were selected with streptavidin-coated DynaBeads® (Life Technologies) followed by a final PCR to amplify the transposon junctions. Size and quantity of DNA in the samples were analyzed on a 2100 Bioanalyzer (Agilent Technologies) before being sequenced.

Two controls were included along with the transposon pooled libraries: one positive control, which contained 16 known mutants from our defined insertion library, and one negative control, which contained NMII genomic DNA without a Tn insertion. Unfortunately, while performing our bioinformatics analysis on the sequencing results, we discovered that our control pools did not work properly. A greater number of genes showed positive in our control pool than were added, and even our negative control yielded positive results. This may be due to PCR bias and the transposon specific primer with the 5' adapter sequence potentially annealing to DNA lacking the transposon ITR sequence. PCR primers and procedures would need to be optimized to prevent false positives from showing in our libraries.

Because the group at Texas A&M AgriLife was inexperienced with these specific type of parameters, we partnered with a group at the University of Exeter, UK, who had successfully completed comparable sequencing experiments with several bacteria [105] and had recently received approval to work with *C. burnetii*. In addition to the control pools and two independent transformation pools, we sent a saturation pool

for sequencing. Through a series of 35 independent transformations, we created a library with near saturation of mutants in the diversity of genes that can be inactivated yet allow the bacterium to remain viable in ACCM. With 500 - 1000 insertions predicted per transformation, this saturation pool would be expected to contain a mutant library of approximately 25,000 independent clones. Sequencing of a genome saturation mutagenesis library could predict essential genes for conditional replication and identify potential virulence factors and targets for antimicrobial therapies.

DNA samples were prepared as described above. The 35 independent transformation pools were combined after recovery and prior to DNA isolation. A portion of these pools was plated on ACCM-agarose to confirm transformation efficiency. Our collaborators at Exeter used a manual TraDIS protocol for library preparations. In brief, the work-flow comprised fragmentation of 5 µg gDNA (or less, if not available) of each mutant library using a Bioruptor® ultrasonicator (Diagenode), followed by a size-exclusion of fragments <150 bp using the GeneRead kit (Qiagen) and DNA purification and quantification. End repair, A-tailing, and adaptor ligation were performed using the NEBNext DNA library prep reagent kit (New England Biolabs), with purification after each step. PCR reactions were optimized to incorporate 5' barcoded adapters for multiplexing, and PCR fragments were size selected on a 2% TBE agarose gel. Finally, the samples were subjected to 20-cycle parallel PCR enrichment for transposon-containing fragments on small amounts of the ligated material to minimize amplification bias. Products were pooled, ethanol precipitated, size-selected on a gel,

and purified before being submitted for sequencing as 100 bp single-end runs on a HiSeq 2500 system at the Exeter Sequencing Facility.

Sequencing resulted in 7-10 million reads per sample. Reads were filtered for the transposon sequence, and then mapped back to the *C. burnetii* RSA493 genome. The number of mapped reads per gene ("hits") was determined for each sample (Table 3). The control pools only contained 16 defined transposon mutants, making them very low-complexity compared to the experimental pools containing all mutants from a single transformation, which potentially contain thousands of mutants. The control pools showed significantly more insertions than expected, suggesting non-specific noise in the system. After increasing the threshold number of hits required for a 'positive' result, 11 of the 16 mutants were successfully identified. Another 3 mutants were potentially positive, while the remaining two were not identified in the control pools. The verified genes all had >1,000 Tn-insertions on average, which therefore might be used as a cut-off point for true signals above the noise level. The two mutants from the control pool that did not show positive after sequencing were CBU_0372 and CBU_2013. Both genes only reached an average of 100 and 50 hits, respectively, which is well below the threshold level of 1,000 hits/gene. It remains to be determined if this could have been due to a problem during the PCR amplification. Moreover, 4 genes with above threshold level of Tn-insertions were identified, which should not have been targeted as no Tn-mutant had been present in the pool. It remains to be elucidated, if the transposon-mutant pools could possibly have been contaminated with transposon mutants in these genes, or if these are true false positives.

Table 3: Results of TraDIS run with six samples. Control pools contained 16 defined mutants. Sample pools 1 and 2 were from single transformations. Saturation pool was a combination of 35 independent transformations.

Sample	# Total Reads	# Filtered reads	# Mapped reads	% Mapped	# hits within CDS	# Genes with Tn
Ctrl pool 1: 16n	7,169,834	7,106,051	4,454,211	62.7	4,454,04	271
Ctrl pool 2: 16n	9,023,281	8,944,037	6,072,066	67.9	6,071,82	136
Ctrl pool 3: 16n	8,823,415	8,746,375	5,947,547	68.0	5,947,336	121
Sample pool 1	9,788,955	9,693,285	1,684,739	17.3	1,609,546	1591
Sample pool 2	9,306,966	9,226,438	6,390,578	69.3	6,326,146	1259
Saturation pool	8,435,688	8,359,340	2,933,113	35.0	2,731,88	180

When applying the 1,000 hits threshold to the three sample pools, which represent transposon mutant pools of unknown identity and complexity, only 7 genes were above threshold in the saturation pool, 6 genes were above threshold in sample pool 1, and 89 genes were above threshold in sample pool 2. Each of the six genes that were identified in sample pool 1, which correspond to CBU_0014, CBU_0021, CBU_0937, CBU_1719, CBU_1720, and CBU_1909, were also identified in at least one other sample, and two of them (CBU_1719 and CBU_1909), were present in all three samples. This could indicate that these genes represent a hotspot for transposon insertions.

Discussion

Understanding host-pathogen interactions and identifying bacterial virulence determinants are essential for developing new therapies to be used against *C. burnetii* infection. The aim of this study was to generate both a library of defined *C. burnetii*

transposon mutants and a pooled transposon library to saturate the non-essential genes of the genome. Transposon insertions were distributed homogeneously throughout the genome, which can be visualized on the genome map. A number of potential T4SS substrates identified in our defined transposon mutant library were characterized by members of our lab [47]. There were a few gaps or regions where no mutations were identified. Some of these gaps were expected since we compared our transposon insertions, which were generated in NMII, to the annotated NMI genome. There is an approximate 26 kb region from the NMI genome that is deleted in the NMII genome. There was a large non-mutated region between CBU_0212 and CBU_0264, which encodes ribosomal proteins that are likely essential for bacterial survival in all conditions. However, we also identified six other regions of about 20 – 30 continuous ORFs each that did not contain mutations. These could be due to mutations in essential genes, or simply areas that we have not identified a transposon insert because mutations do not approach saturation.

We optimized the rescue cloning protocol to an efficiency of nearly 60%. Unfortunately, we failed to adapt touchdown PCR to work with our transposon mutagenesis system. Future studies should continue to pursue this approach, as we achieved approximately 15 – 20% success rate for defining clones (Table 4). New primers should be designed to include random, universal, and nested *Himar* primers combining different primer combinations and running multiple PCR reactions to achieve the greatest success [106]. Not all clones are successfully rescue cloned, but by

optimizing the touchdown approach, these two methods could be combined to allow for significantly higher throughput for defining transposon insertions.

Table 4: Comparison of rescue cloning and Touchdown PCR methods for isolating and sequencing transposon insertions.

Rescue Cloning	Touchdown PCR
Digest isolated gDNA	PCR isolated gDNA using non-specific degenerate primer
Ligate	PCR clean-up
Transform into *E. coli*	Sequence
Isolate plasmid	
Sequence	
60% efficiency, but time consuming and costly	*Currently only 15% efficiency*

A near saturation mutant genome pool could prove extremely beneficial for identifying virulence determinants in *C. burnetii*, but there would still be a subset of genes not represented in our mutant libraries. The genes in these non-mutated regions will be characterized as essential to *C. burnetii's* survival in growth conditions (ACCM) used to culture mutants. We generated 50 pools of transposon mutants from independent transformations with pKM225. These pools were tested for viability and transposon insertion. However, these pools are still undergoing testing to more accurately predict the number of ORF insertions in each pool. We combined 35 of these pools into one genome saturation pool and provided the purified DNA to our collaborators at the

University of Exeter. We obtained our first sequencing data from this saturation pool along with two individual pools and three control pools.

We were unable to achieve a reliable readout using either the HITS or TraDIS approach. As an alternative approach, the next attempts could perform transformations and plate them on solid medium instead of maintaining liquid culture. Colonies could then be picked and pooled into one library, ensuring a greater viability of clones in the pool. Another possible improvement could be to use enzyme digestion for DNA fragmentation than random shearing. This would allow for more specificity and greater efficiency in isolating the Tn junctions. A major problem that we encountered was with our positive control pools. The sequencing reads were producing some false positives as well as false negatives. About 75% of the clones in the pool were positively identified, yet with large variations in apparent copy number. Of the 16 transposon mutants, 14 were successfully identified, but there were also false negatives. Previous studies utilizing these approaches did not include a positive control pool [99-101]. It could be that the Illumina sequencer is not designed to accurately read such a small number of products in a sample. The software needs a certain amount of diversity in order to accurately differentiate between sequences. This system discerns hundreds of thousands of clones but may become 'confused' with less than 20. Instead of using a positive control pool, false positive results could be addressed by repeating the sequencing as multiple replicates. Studies into analyzing the genome saturation pool by deep sequencing should continue, as the readouts will prove extremely beneficial to *Coxiella* research.

CHAPTER III

DEVELOPING VIRULENCE RELATED SCREENING AND CHARACTERIZATION APPROACHES FOR SPECIFIC TRANSPOSON MUTANTS

Background

The identification of additional *C. burnetii* virulence factors using recently developed genetic techniques will be instrumental in finding targets for new therapies. The aim of this study was to develop methods to identify a set of basic structure and metabolic related genes that are required for survival and replication in ACCM, *in vitro,* and *in vivo*. The mouse macrophage-like cell line, J774.A1, mouse fibroblast cell line, L929, and human epithelial cell line, HeLa, were used for *in vitro* studies. There are several animal models of virulent phase I *C. burnetii* infection available, including guinea pig, mouse and primate models; however, SCID mice are the only animal model to enable high replication of *C. burnetii* NMII [81,82].

We predict many genes are required for *C. burnetii* survival and replication in cells or in animals while having no phenotype in ACCM-2, and that many of these genes are critical for its pathogenic process. The genes required for replication in cells likely include those that contribute to attachment, invasion, formation of a suitable replication niche, and persistence. The genes uniquely required for infecting animals may include those responsible for immune modulation or evasion. We intended to use the pooled mutant library to compare insertion mutant complexity between input and output pools from *in vitro* infected cells (J774.A1 or L929) or infected SCID mice; however, since the

methods for analyzing these pools are still being optimized, we have relied on the defined clonal mutants to evaluate phenotypes in this thesis. A high-throughput method for screening transposon mutants will be essential for comprehensive identification of virulence related genes since screening individual clones is time intensive and expensive, particularly in animals.

To test the hypothesis that a subset of genes will prove attenuated in one or more stages of the pathogenic process, bacterial clones containing Tn insertions were compared to NMII in tissue culture cells and a mouse model of infection. The growth rate and ability to infect and replicate was monitored. Additional studies on phenotypes of specific clones were prioritized using bioinformatic prediction of function for specific mutations. The results of these combined *in vitro* and *in vivo* screens provide a wealth of new data for future studies in an effort to understand the critical pathways essential for this pathogen.

Materials and Methods

Bacterial Strains, cell lines, and growth conditions

Escherichia coli strain DH5α was propagated at 37 °C in Luria-Bertani (LB) broth or agar, purchased from Difco (Sparks, MD). Media were autoclaved to 121 °C for 30 minutes and supplemented with the appropriate filter sterilized antibiotic(s) at the final concentrations listed: chloramphenicol (Cm) (34 μg/ml) or kanamycin (Kan) (50 μg/ml). Broth cultures were usually grown overnight (O.N.) in 5 ml of LB and shaken at 200 rpm. Select strains were stored at -80 °C after resuspending 5 ml of O.N. culture in 1 ml of autoclaved glycerol (10% v/v) in LB without antibiotics.

The laboratory derived *C. burnetii* Nine Mile phase II (NMII), strain RSA 439 clone 4, was cultivated in T-75 cell culture flasks or 0.2-µm-pore-size-filter-capped 125-ml Erlenmeyer flasks containing 75 ml of medium or T-25 flasks containing 20 ml of medium grown in filter sterilized liquid ACCM-2 or ACCM-agarose. Cultures were grown for approximately 7 days at 37 °C in a 2.5% O_2 and 5% CO_2 environment. Oxygen was displaced by nitrogen gas. Where required, Cm was used at 5 µg/ml.

J774A.1 cells were cultured in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% fetal bovine serum (FBS). Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% FBS was used for culturing L929 cells. All cell lines were maintained at 37 °C with 5% CO_2.

Real time quantitative PCR

Tissue and cells were lysed with 200 µl lysis buffer (1M Tris, 0.5M EDTA, 7 mg/ml glucose, 28 mg/ml lysozyme) and 10 µl proteinase K (20 mg/ml) and incubated O.N. at 60 °C, followed by the addition of 21 µl 10% SDS and incubated at room temperature for 1 h. DNA was extracted using High Pure PCR Template Preparation Kit (Roche Molecular Biomedicals, Indianapolis, IN), and stored at -20 °C until use. Real time PCR (rtPCR) was performed using an Applied Biosystems 7500 Real time PCR System. The recombinant plasmid DNA, containing the *IS1111* gene, was used as standard DNA to quantify copy numbers.

Growth of transposon mutants in media

To rule out general growth defects, growth curves in ACCM-2 were conducted by inoculating 20ml ACCM-2 with $1.0x10^6$/ml of CB439, an intergenic mutant, or

specific transposon mutants. At 1d, 4d, and 7d post-inoculation, 1ml of culture was removed, pelleted, and resuspended in 200µl tissue lysis buffer with 10µl proteinase K [107]. DNA was isolated from the ACCM-2 cultures using High Pure PCR Template Prep Kit (Roche) per manufacturer's instructions and purified DNA was quantified using qPCR with TaqMan and primers specific for IS1111 [108].

Growth of transposon mutants in vitro

Individual *C. burnetii* NMII defined mutants were used to infect HeLa, J774.A1, L929, or MH-S cells. Cells were seeded at $1.25x10^4$ cells/well (96-well plate) or $5x10^4$ cells/well (24 well plate) and infected at a multiplicity of infection (MOI) 100 for 4h. An intergenic mutant or mCherry expressing *C. burnetii*-pKM244 was used as a positive control. Concentration of *C. burnetii* strains axenically cultured in ACCM-2 was determined using qPCR with *IS1111*gene specific primers [108]. Four hours post-infection (HPI), cells were washed 3 times with 1X PBS to remove un attached bacteria, fresh media was added to each well, and cultures were incubated at 37 ℃ with 5% CO_2. At 1d, 4d, and 7d, cells were scraped and pelleted cells were resuspended in tissue lysis buffer with 10µl proteinase K [107]. DNA was isolated from the infected cells using High Pure PCR Template Prep Kit (Roche) per manufacturer's instructions and purified DNA was quantified using qPCR with TaqMan and primers specific for *IS1111* [108].

High-throughput screening of transposon mutants in vitro

An alternative method to screen the large numbers of defined insertion clones *in vitro* is to image infected cells with a Nikon-A1 Confocal Microscope System (Nikon, Tokyo, Japan), fluorescence image or a Cytation3 cell imaging microplate reader

(BioTek, Winooski, VT) for detection of mCherry expression (594nm). J774.A1, L929, or MH-S cells were seeded in 48 or 96 well plates and infected with individual mutants in triplicate. Four HPI, cells were washed and fresh media +Cm was added. Cells were grown for 7d, fixed, stained, and analyzed. Vacuole size and number per cell were used to determine efficiency of infection compared to wild type.

Growth in vivo

SCID mice were infected with individual C. burnetii defined transposon mutants and monitored for bacterial dissemination and clearance. As previously shown by our lab, SCID mice (deficient in T and B cells) are susceptible to infection with the low virulence C. burnetii NMII strain [82]. Five – 6 week old SCID mice were infected by intraperitoneal (IP) route with 10^6 bacteria (100 ul). As control infections, mice were infected with: wild type NMII strain (positive); a Himar1-containing isolate, which has inserted into an intergenic region and was determined not to have a growth defect in vitro (positive); or PBS buffer (negative). Clinical signs were observed and body weight measured daily. Mice were maintained for 14 days, sacrificed, and lung and spleen tissues obtained for DNA isolation and quantification by qPCR. Spleens were collected and weighed for determination of splenomegaly. Infected spleen and lung tissues were homogenized in 1mL ddH_2O. To isolate DNA from tissues, 100 µl homogenized tissue was added to 900 µl urea or tissue lysis buffer (Roche) with 100 µl proteinase K. DNA was isolated from the infected cells using High Pure PCR Template Prep Kit (Roche) per manufacturer's instructions and purified DNA was quantified using qPCR with TaqMan and primers specific for IS1111 [108].

Statistical analysis

Statistical analyses were performed using a one-way analysis of variance (ANOVA) and Prism software (GraphPad Software, Inc., La Jolla, CA).

Results

Defined transposon mutant library in vitro

The defined transposon mutant library contains approximately 800 clones in nearly 400 unique ORFs including roughly 60 insertions in *dot/icm* genes. The T4BSS is a known virulence factor of *C. burnetii* [35], and studies have shown Tn insertions in *icm* gene loci are defective for intracellular replication [32,69]. One of the first clones we identified in our library was the T4SS component, *icmX*. We tested this *icmX*::Tn mutant *in vitro* to demonstrate our methods for generating and characterizing defined transposon mutants.

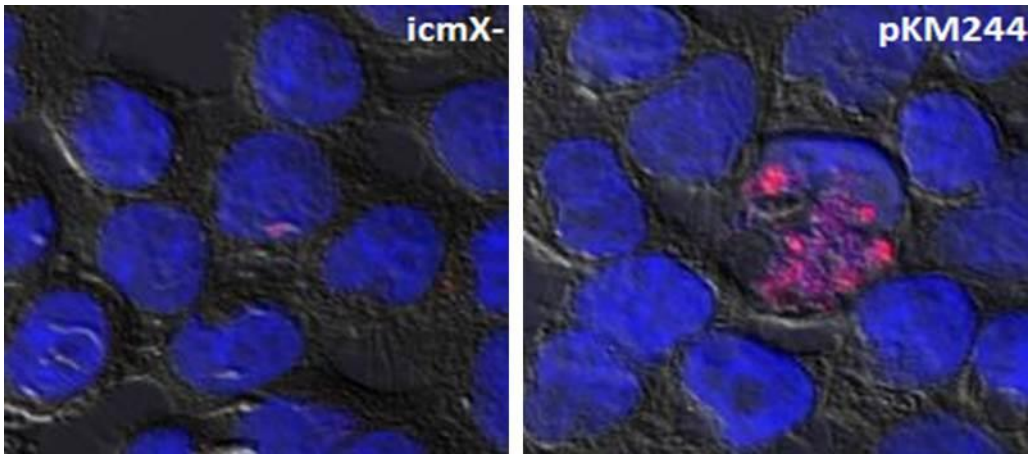

Figure 9: Growth of icmX::Tn mutant in HeLa cells. A T4SS component mutant, *icmX*, was isolated using the *mariner*-based *Himar1* transposon system. HeLa cells were infected at an MOI of 100 with each strain and growth was monitored at 7d. The *icmX* mutant displayed diminished growth and reduced vacuole size and bacterial load compared to the wild type, mCherry-expressing *C. burnetii*-pKM244.

HeLa cells were infected at an MOI of 100 with the *icmX*::Tn mutant or *C. burnetii*-pKM244, and growth was monitored for 7 days. The pKM244 plasmid transformed into *C. burnetii* gives the bacteria Cm resistance and mCherry expression for fluorescent observation. The *icmX* mutant displayed diminished growth and reduced vacuole size and bacterial load compared to wild-type (Figure 9).

This demonstrated our strategy for generating transposon mutants was successful, and we could continue screening the library. However, it would be too expensive and time prohibitive to quantify 800 clones for infection using these methods. Until a high-throughput method was developed, we prioritized our efforts using bioinformatic prediction of function for specific mutations. T4SS components were tested and characterized by members of our lab using the defined transposon mutants [47]. Other members of our lab are studying potential T2SS components and the production of type IV pili and have used *pilD*::Tn and *pilE*::Tn mutants generated in our library to determine secretion phenotype. Work is also being done to characterize an *enhA, B, and C* ::Tn mutants. Enhanced entry proteins have been shown to be involved in entry of *L. pneumophila* into host cells, as mutations in the *enhC* gene resulted in a significantly reduced-entry phenotype [109]. Because of the similarity between these two organisms, we would predict a similar phenotype in *C. burnetii.*

As a method of screening more mutants at once, we seeded L929 fibroblasts in 24 well plates at 10^5 cells/ml and infected with comparatively prepared bacteria in 0.5 µl. This initial screen was conducted with 66 mutants. Cultures were incubated for 7 days before being fixed and analyzed by confocal microscopy. This resulted in 12 mutants

with a potential growth defect: CBU_0007, CBU_0053 (*enhA*), CBU_0062 (*dnaJ*), CBU_0064 (*parE*), CBU_0072 (*ankA*), CBU_0206, CBU_0571, CBU_0661, CBU_0910, CBU_0945, CBU_2082, and an intergenic mutant before CBU_1050. These 12 mutants were then quantified by qPCR, and cells were infected on coverslips at MOI 100 in triplicate. However, no growth defect was observed with these controlled infections. The phenotypes seen initially may be due to such a small volume of bacteria being added to each well.

This experiment was repeated using 170 hypothetical proteins in the defined transposon mutant library. These mutants were first grown in 100 µl ACCM in 96 well plates. L929 cells were seeded in 96 well plates (1.25×10^4 cells / well). Cells were then inoculated with 2 µl mutant for 4 h. Cells were washed to remove unattached bacterium and then fresh medium was added and cells were incubated for 7 days. After infection, cells were fixed, stained, and analyzed for growth phenotypes on a confocal microscope or a Biotek Cytation 3 Imaging Reader (Figure 10).

We screened these mutants on the confocal microscope using a program to conduct automated analysis. Unfortunately, it was too sensitive to autofocus the wells in the plastic tissue culture dishes we were using. The confocal software would be better able to maintain autofocus on glass bottom plates. Another problem we ran into with this experiment was the number of tissue culture cells. By day 7, the wells were nearly saturated. Seeding at a lower number of cells per well or in a greater volume in 48-well plates would likely solve these issues.

The Biotek Cytation3 Imaging Reader is a fully automated method of analyzing vacuole size and number, and relative fluorescence in each well to efficiently obtain visual as well as quantitative fluorescence readouts that can easily be compared between wells. The first sample shown below, CBU_0197, had an average fluorescence intensity of 36,000 (TexasRed), which was comparable to wild type (data not shown). The next sample, CBU_1071, showed a reduced growth phenotype, with an average fluorescence intensity of 23,000. Finally, the third sample, CBU_0041, has a no growth phenotype, and gave an average fluorescence intensity of only 7,000. This is in agreement with data previously reported for CBU_0041 [47].

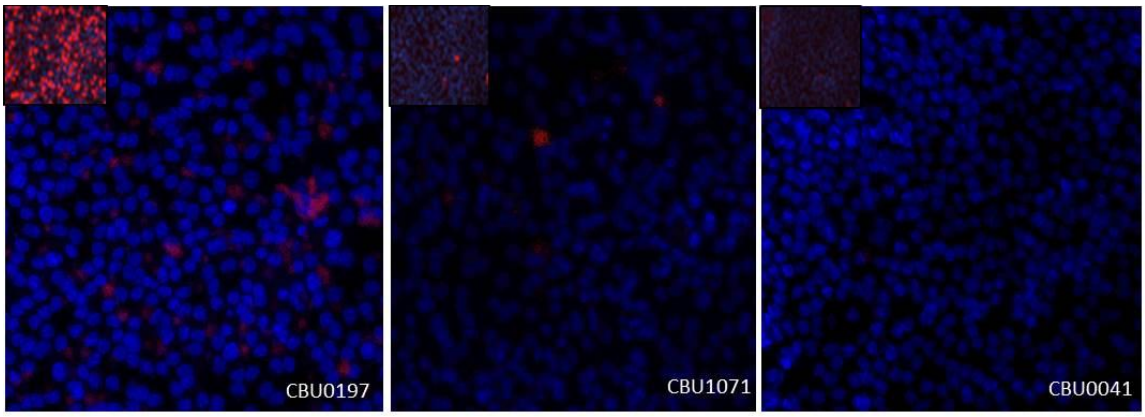

Figure 10: Growth of transposon mutants in L929 cells. A screen of over 150 Tn mutants yielded growth defects in a number of samples. Cells were analyzed 7 days post infection using the BioTek Cytation3 Imaging Reader (inset) or by confocal microscopy.

Defined transposon mutant library in vivo

After identifying mutants with reduced growth phenotypes *in vitro*, we assessed these mutants *in vivo*. We infected 5-6 week old SCID mice by intraperitoneal injection

with: NMII, an intergenic Tn mutant (positive control), a T4BSS component mutant (*icmX*), four Tn mutants predicted to be T4BSS substrates (CBU_2052, CBU_0041, CBU_0937, and CBU_0425), or a T4P component mutant (*pilD*). All of these mutants except the intergenic and pilD have clear cell growth phenotypes. Organs were harvested 14 DPI and spleen weight was compared as a percentage of total body weight for each animal to determine splenomegaly (Figure 11). Mice infected with *wt* NMII or the intergenic Tn mutant showed severe splenomegaly. However, no splenomegaly was seen in mice infected with the other Tn mutants.

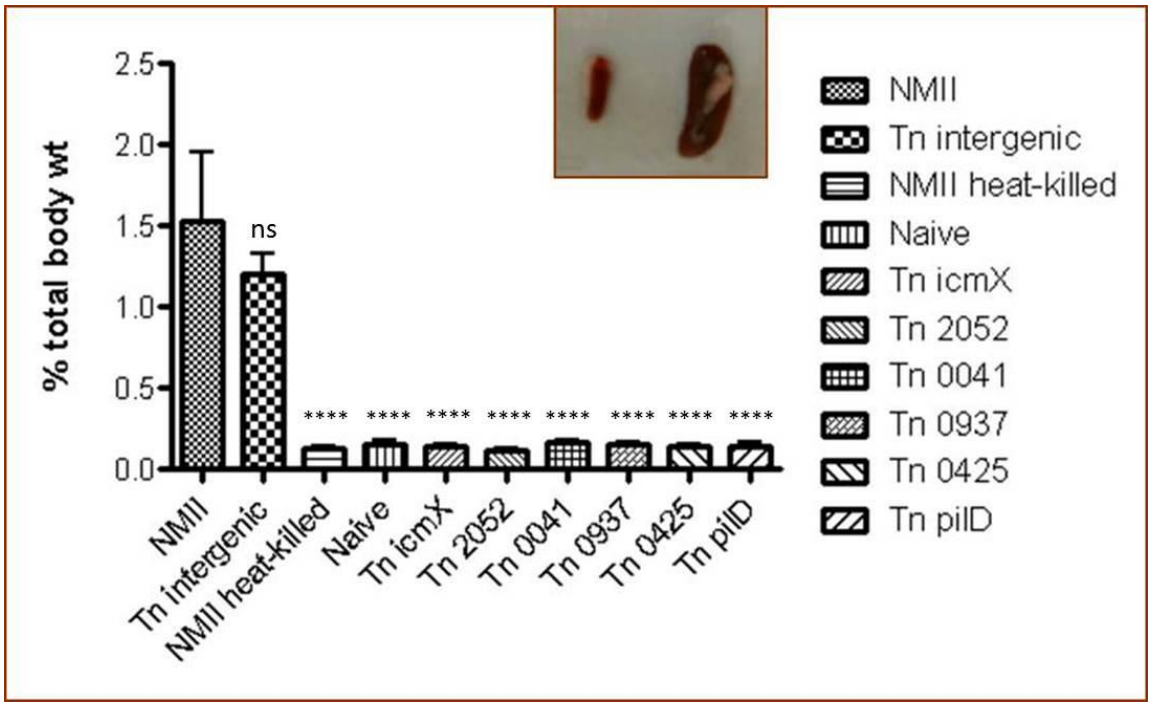

Figure 11: *In vivo* model of infection established for NMII strain. SCID mice were infected with *wt* or killed NMII or a series of Tn mutants, including an intergenic mutant. At 14 DPI, splenomegaly was detected in mice infected with both *wt* NMII and the intergenic mutant. The remaining Tn mutants caused little increase in spleen size and were comparable to the naïve control group. 'ns': $P > 0.05$; '****': $P \leq 0.0001$.

After spleens were weighed, they were homogenized in 1 ml ddH_2O. One hundred µl of the liquefied organ was used for DNA isolation and quantification of bacterial copy number by qPCR (Figure 12). As expected, the amount of bacteria found in the spleens correlated with splenomegaly. Spleens of mice infected with *wt* NMII or the intergenic Tn mutant contained levels of bacteria greater than the infected dose.

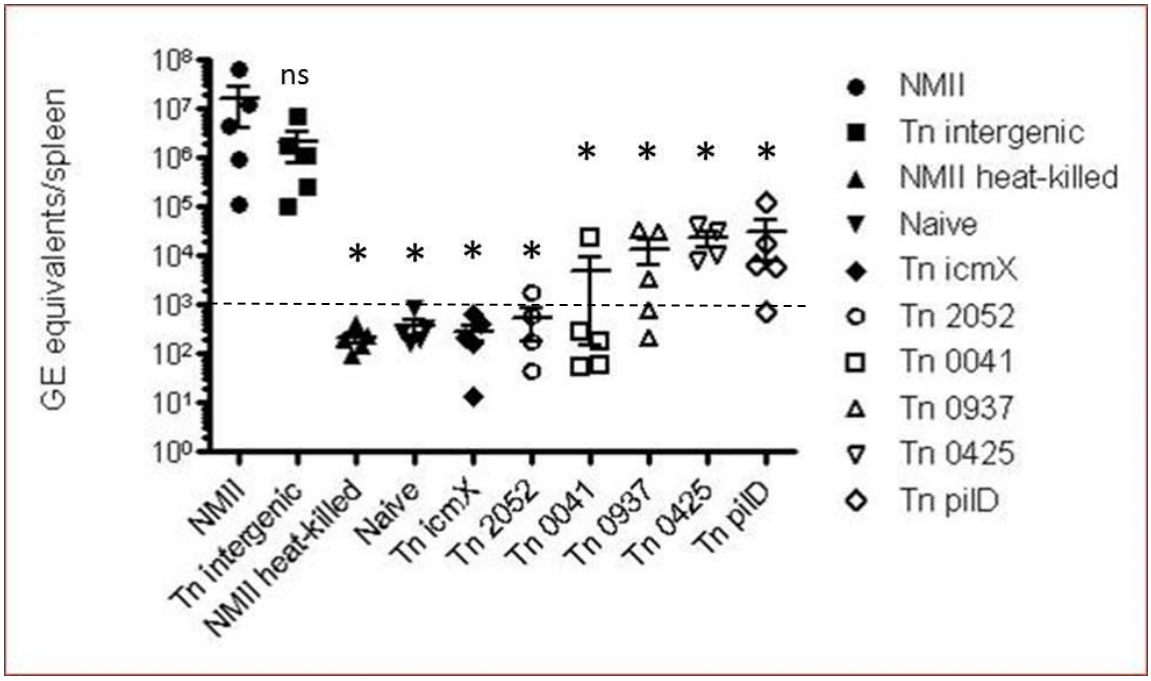

Figure 12: Growth of transposon mutants *in vivo*. SCID mice were infected with 10^6 copies of *wt* NMII or Tn mutant strains. Spleens were harvested 14 DPI and analyzed for genome copy by qPCR. Both the *wt* and intergenic mutants showed high bacterial loads, while mutant strains with insertions in *C. burnetii* ORFs displayed growth reduced by 2-4 logs, some as low as the uninfected control. 'ns': $P > 0.05$; '*': $P \leq 0.05$.

We did not recover detectable levels of bacteria from spleens of mice infected with the *icmX*, CBU_2052, or CBU_0041 Tn mutants, similar to naïve animals. Growth of bacteria was present but reduced in spleens of mice infected with CBU_0937,

CBU_0425, and *pilD* Tn mutants. The T4SS substrates CBU_0041, CBU_0937, CBU_0425, and CBU_2052, were characterized by members of our lab and determined to be crucial for intracellular replication and CCV formation, and were therefore renamed *cirA*, *cirB*, *cirC*, and *cirD* (*Coxiella* effector for intracellular replication), respectively [47].

Discussion

Transposon mutagenesis is an extremely valuable tool for studying mechanisms used by bacterial pathogens to evade the host immune system. The previously generated *C. burnetii* defined transposon mutant library was used to map *C. burnetii* genes essential for growth and viability. To determine which transposon mutants to begin our analysis with, we used bioinformatic prediction of function to identify specific genes suspected to encode virulence factors as defined by Koch's postulates. We identified nearly 60 insertions in T4SS components (*dot/icm* genes), another 20 in T4SS substrates [47], as well as insertions in genes predicted to be involved in type IV pilus assembly (*pil* genes) and entry (*enh* genes). These mutants - most of which have displayed growth defects *in vitro*, *in vivo*, or both - have been, and continue to be, extremely beneficial in advancing research in our lab.

High-throughput screens are the most effective way to globally identify and characterize novel bacterial virulence determinants. Using efficient screening methods to analyze clonal transposon mutant libraries should provide a wealth of new knowledge that will dramatically improve our understanding of how this pathogen interacts with its host. The identification of additional *C. burnetii* virulence factors using recently

developed genetic techniques will be instrumental in finding targets for new therapies. The aim of this study was to develop methods to screen a library of *C. burnetii* transposon mutants and identify a set of basic structure and metabolic related genes that are required for survival and replication. We proposed two tools for screening host cells infected with transposon mutants *in vitro*. Both methods involve infecting cells in 48- or 96- well plates and imaging with a high sensitivity automated digital microscopy system to detect fluorescence levels and determine vacuole size and number.

The first system uses an A1 confocal microscope imaging system, which is currently available in our lab. The benefits of this system are the ability to take high resolution images with software that can automatically focus, detect fluorescence, take multiple images per well, and move from one well to the next. Unfortunately, the high sensitivity and high resolution of this machine are also a drawback. The slightest tilt in the tissue culture plate will prevent the microscope from focusing in the proper plane, precluding detection of the cells. Because this is often a problem with plastic tissue culture plates, the confocal system requires use of more costly, glass bottom dishes to function at its highest potential. Also, given the small size of *C. burnetii,* 0.2 – 1 μm long [27], the Nikon software requires each well to be imaged at a magnification of at least 40 or 60x, requiring more images, and thus more time, per well.

The second system for *in vitro* imaging of cells infected with transposon mutants uses a Cytation3 cell imaging microplate reader. This is a very high throughput system that could potentially result in significant time savings when screening an entire transposon mutant library. BioTek is a microplate instrument company, and this machine

has been optimized to automatically and efficiently move from well to well. The Cytation3 also offers autofocus, auto exposure, and auto LED intensity. It also includes a Hit Picking protocol--with built-in plate reader, where the plate can be read first to get RFUs of each well and then image only those wells of interest with high enough fluorescence intensity values. This also cuts down on data storage amount. Another big advantage of the Cytation3 is the ease of use. The user interface is friendly enough for untrained users to capture images or read a plate without first going through lengthy training.

Each of these systems can be extremely advantageous as a high throughput method for analyzing infections with Tn mutants. Another option could be to combine the two systems, first imaging the entire plate on the Cytation3, then analyzing specific wells at a higher resolution using the confocal microscope. The Cytation3 could serve as the workhorse, even by inexperienced users, due to its speed and ease of use. This would save the confocal for specific imaging and experienced users, and help prevent damage to confocal, which is usually expensive to maintain. Any phenotype observed on one of these systems would then be further characterized by quantifiable infection studies to determine difference in uptake, growth rate, CCV formation, or other phenotypes.

Screening a library of defined transposon mutants with a digital imaging system is a valuable tool that can be used to identify growth phenotypes *in vitro;* however, there is still a need for a high-throughput method of screening these isolated mutants *in vivo.* Recently, an insect model of *C. burnetii* NMII infection was developed. This model was used to characterize T4BSS mutants in an *in vivo* system. This is the first non-

mammalian *in vivo* model of *C. burnetii* infection and is potentially suitable for rapidly characterizing mutant phenotypes [84].

Using pools of random transposon mutants is a well-established method of efficiently identifying genes essential for bacterial invasion and replication in the host [110]. A *Himar1* transposon system is used to generate a pool of random mutants. This mutant pool is then subjected to various growth conditions or infection models and surviving mutants are recovered in an output pool. The insertion site of the transposon in each mutant in the input and output pools are identified by next-generation sequencing and compared to each other. Genes in which no transposon insertions are being detected in the output pool are considered to be essential for a defined growth or virulence condition. This would be an extremely useful tool for identifying genes essential for growth *in vitro* and *in vivo*, since nearly all genes could be analyzed in a single well or animal.

A thorough understanding of *C. burnetii* virulence factors is lacking. Major advances in *Coxiella* research in recent years allow for high throughput screens of large libraries of transposon mutants. This study establishes methods for evaluating libraries of defined Tn mutants and pools of Tn mutants, which will ultimately lead to the identification of bacterial genes involved in host/pathogen interactions.

CHAPTER IV

SUMMARY AND CONCLUSIONS

C. burnetii is a Gram-negative bacterium that causes the zoonosis Q fever. The disease, which has been described in nearly every country worldwide, was first identified almost 80 years ago. Despite this, its virulence mechanisms are poorly understood [98]. The organism's intracellular lifestyle has been a major roadblock in the field of *Coxiella* research, but recent advances have helped researchers overcome these hurdles and significant progress has been achieved in the past few years. These tools should allow identification and characterization of specific mechanisms of *C. burnetii* pathogenesis [4].

The long range goal of this study was to generate transposon mutant libraries in *C. burnetii*, and then use these libraries to identify genes essential for growth *in vitro* and *in vivo*. The broad objective was to develop methods to identify a set of basic structure and metabolic related genes that are required for survival and replication. Recent advances in growth medium and the development of a single plasmid *Himar1* transposon system by our lab supported the hypothesis that a large collection of specific clonal mutations in *C. burnetii* could be isolated and screened for their contribution to the pathogenic process *in vitro* and *in vivo*. Although we did not approach saturation of the genome with our defined Tn mutant library, we optimized methods to generate defined transposon mutants, resulting in mutations in nearly 20% of the predicted ORFs. Included in these mutants were a number of bioinformatically predicted virulence factors

that we further studied in cells and an animal model of infection. Our findings are consistent with previous studies that demonstrate the Dot/Icm T4BSS is essential for generating and replicating in a large PV [32,47,69,97].

We also developed methods for high throughput screening of Tn mutants *in vitro* by imaging on either a Nikon confocal microscope or a BioTek Cytation3 imaging system. The confocal is more sensitive and higher resolution but requires more time and technical proficiency. The Cytation3 does not produce high resolution images but is easy to operate and can analyze an entire microplate very quickly. Similar methods for screening *C. burnetii* Tn mutants have recently been reported [53,111].

We generated a genome saturation transposon mutant pool by combining transposon mutant pools from 35 independent transformations. With each transformation expected to generate 500-1000 Tn insertions, this should be more than sufficient to saturate all of the non-essential ORFs in the *C. burnetii* genome. Although we were unable to analyze the level of genome saturation in the random transposon mutant pools by deep sequencing, significant steps were made to optimize this procedure. The first step in the library preparation protocol may need to be updated to use enzyme digestion instead of an ultrasonicator to shear the DNA. More specificity might also be achieved by redesigning the primers and using only 1 adaptor instead of 2. Finally, using needle aspiration on output pools to release bacterial DNA from eukaryotic host DNA would provide a more pure pool for sequencing. The identification of additional *C. burnetii* virulence factors using these recently developed genetic techniques will be instrumental in finding targets for new therapies.

Future directions

Clonal transposon mutants should continue to be generated to approach saturation of all non-essential ORFs in the defined Tn mutant library. In addition to rescue cloning, touchdown PCR should be used to identify isolated clones. New or additional semi-random primers may be designed and try different primer combinations for different clones before this procedure is successful. It may also be advantageous to screen every isolated clone, even before identifying the Tn insertion site. This would allow us to detect mutants from the library based on their interesting phenotypes and prioritize efforts to identify these clones first. It may be beneficial to explore the recently described insect model of *in vivo* infection as a means to efficiently screen Tn mutants.

In addition, pools of transposon mutant libraries are a valuable tool to efficiently identify genes essential for bacterial invasion and replication in the host. The procedure for analyzing these libraries is nearly optimized, and then we will be able to identify essential genes by this method as well. Genes essential for the innate immune response would not be expected to show a growth defect *in vitro* but should prove attenuated in the SCID mouse model. It would not be feasible to test every clonal mutant that replicates normally in cells, individually in a SCID mouse. Therefore, comparing input and output pools by TraDIS is the most efficient means of identifying innate immune genes.

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APPENDIX A

LIST OF BACTERIAL STRAINS AND PLASMIDS USED IN THIS STUDY

Strain / Isolate	**Description**	**Source**
C. burnetii RSA439	Phase II, Clone 4	Montana, tick, 1935
E. coli DH5α	*F′(Φ80dΔ(lacZ)M15), recA1, endA1, gyrA96, thi1, hsdR17 (rk-mk+), supE44, relA1, deoR, Δ(lacZYA-argF),U169*	Stratagene
pKM225	*pMW1650, com1p-TnA7, groESp-mCherry, com1p-cat, CmR*	K. Mertens [95]
pKM244	*pJB908a, groESp-mCherry, com1p-cat, CmR, AmpR*	K. Mertens [95]

APPENDIX B

LIST OF PRIMERS USED IN THIS STUDY

Name	Sequence	Experiment
catF	5’-GTACTGCGATGAGTGGCAG-3’	Rescue Cloning
ColE1R	5’-CTTTCCTGCACTAGATCCCC-3’	Rescue Cloning
HIB17	5’-CGGAATTCCGGATNGAYKSNGGNTC-3’	Touchdown
Himar1-F1	5’-ACGACGCTCTTCCGATCTGAATGCGGGGACTTATCAGCCAACC-3’	HITS
Himar1-F2	5’-ACGACGCTCTTCCGATCTCTTACCGGGGACTTATCAGCCAACC-3’	HITS
Himar1-F3	5’-ACGACGCTCTTCCGATCTTCGCTCGGGGACTTATCAGCCAACC-3’	HITS
Himar1-F4	5’-ACGACGCTCTTCCGATCTAGCGACGGGGACTTATCAGCCAACC-3’	HITS
INDX15	5’-CAAGCAGAAGACGGCATACGAGATcaggtcgGTGACTGGAGTTCAGACGTG*T-3’	HITS
Adaptor 1	5’-GATCGGAAGAGCACACGTC*T-3’	TraDIS
PCR 1 adaptor	5’-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3’	TraDIS
MPX1	5’-CAAGCAGAAGACGGCATACGAGATATCACGGTGACTGGAGTT*C-3’	TraDIS
MPX2	5’-CAAGCAGAAGACGGCATACGAGATCGATGTGTGACTGGAGTT*C-3’	TraDIS
MPX3	5’-CAAGCAGAAGACGGCATACGAGATTTAGGCGTGACTGGAGTT*C-3’	TraDIS
MPX4	5’-CAAGCAGAAGACGGCATACGAGATTCAGATCGTGACTGGAGTT*C-3’	TraDIS
MPX5	5’-CAAGCAGAAGACGGCATACGAGATACAGTGGTGACTGGAGTT*C-3’	TraDIS
MPX6	5’-CAAGCAGAAGACGGCATACGAGATACTTGAGTGACTGGAGTT*C-3’	TraDIS
MPX7	5’-CAAGCAGAAGACGGCATACGAGATTAGCTTGTGACTGGAGTtC-3’	TraDIS
MPX8	5’-CAAGCAGAAGACGGCATACGAGATGATCAGGTGACTGGAGTtC-3’	TraDIS
Himar-PCR-3	5’-AATGATACGGCGACCACCGAGATCTACACAGTCAGTTATTGGTACCCTTAAAC*G-3’	TraDIS
Himar-seq2	5’-CAGACCGGGGACTTATCAGCCAACC-3’	TraDIS