

DISCOVERY OF GENETIC CORRELATES IMPORTANT FOR CHLAMYDIA INFECTION
AND PATHOGENESIS

By
Kelly S. Harrison

Submitted to the graduate degree program in Molecular Biosciences and the Graduate Faculty of
the University of Kansas in partial fulfillment of the requirements for the degree Doctor of
Philosophy.

Chairperson (P. Scott Hefty, Ph.D.)

*

(Lynn E. Hancock, Ph.D.)

*

(Susan M. Egan, Ph.D.)

*

(David J. Davido, Ph.D.)

*

(Mario Rivera, Ph.D.)

*Committee Members

Date Defended: July 5th 2017

The Dissertation Committee for Kelly S. Harrison certifies that this is the approved version of the following dissertation:

DISCOVERY OF GENETIC CORRELATES IMPORTANT FOR CHLAMYDIA INFECTION
AND PATHOGENESIS

Chairperson (P. Scott Hefty, Ph.D.)

Date Approved: July 25th, 2017

ABSTRACT

Chlamydia species are responsible for over 1.2 million reports of bacterial sexually transmitted infections in the United States; a number that has been steadily increasing for the past decade. Worldwide, a cumulative 131 million new cases of *Chlamydia trachomatis* are estimated among individuals between ages 15-49. In most individuals, chlamydial infections are asymptomatic, resulting in long-term sequelae such as pelvic inflammatory disease, salpingitis and infertility. Along with genitourinary infections, *Chlamydia* is also the leading cause of blinding trachoma, affecting nearly 1.9 million people across 42 different countries. While current treatment with antibiotics remains successful in combating infections, evidence of persistent infections, acquisition of antibiotic resistances, and recurring exposure intensifies the necessity for enhanced prophylactic approaches, including the development of a vaccine. In order to develop these advances, species-specific targets, as well as mechanisms the bacterium uses to establish infection must be identified. My research focuses on two forward-genetic approaches to identifying these correlates of pathogenicity: homologous recombination and transposon mutagenesis.

Majority of information pertaining to *Chlamydia*'s pathogenicity in a mammalian host has been derived from the mouse-adapted species, *Chlamydia muridarum*. This species readily infects the vaginal canals of female mice and ascends to the upper genital tract—the primary site for causing disease in women. Conversely, the human-adapted strain, *C. trachomatis*, is incapable of ascension in the mouse model of infection and rapidly cleared. Even with the drastic differences in pathogenicity, *C. muridarum* and *C. trachomatis* share ~98% genome sequence identity, with most of the genetic differences occurring within the plasticity zone. In our efforts

to understand which genetic components are responsible for ascension, *in vitro* recombination between *C. trachomatis* and *C. muridarum* was completed, enriching for recombination around this plasticity zone. I used molecular approaches to characterize regions of crossover among the hybrid progeny, focusing on hybrids containing *gyrA-2* from *C. muridarum* as an indicator for genetic exchange near the plasticity zone. Among the 35 hybrids assessed, only three showed integration of the large cytotoxins specific to *C. muridarum*: 4357, 4406, and 4537. These three recombinants integrated only two of the cytotoxins, *TC0438* and *TC0439*; none of the samples showed integration of the third cytotoxin, *TC0437*. To assess whether *TC0438* and *TC0439* are responsible for *C. muridarum*'s ability to ascend to the UGT, I used the mouse vaginal model of infection with these hybrids. Three mice each were infected with the hybrids and uterine horns isolated for assessment of ascension. Seven days post-infection, only one animal, infected with isolate 4406, showed detectable *Chlamydia* in the uterine horns. No other animals from any of the hybrids showed detectable organisms in the uterine horns, suggesting these two cytotoxins are not sufficient to cause a gain-of-function. The remaining 32 hybrids showed recombination at the same intergenic site between the *C. trachomatis trpA* and *C. muridarum TC0440*. This region may indicate a recombination hotspot or chi site within *Chlamydia* or may highlight the selective pressure against *C. trachomatis* to harbor the cytotoxins.

Secondly, the development of a transposon system functional in *Chlamydia* permitted the analysis of single-gene disruptions and their roles in growth and development. I used Tn libraries generated in both *C. trachomatis* and *C. muridarum* to characterize *in vitro* and *in vivo* phenotypes. All 13 Tn mutants from *C. trachomatis* were single insertions while three of the 26 *C. muridarum* mutants showed double inserts. One mutant from *C. trachomatis*, *CT696::Tn*, had a significant defect in growth *in vitro* resulting in lack of inclusion formation and aggregation of

RBs. *In vivo*, two mutants from each library, *CT148::Tn* and *CT868::Tn* from *C. trachomatis*, and *TC0657::Tn* and *TC685::Tn* from *C. muridarum* showed decreases in bacterial burden in the uterine horns of mice.

In addition, I performed *in silico* analysis of the Tn insert within the hypothetical protein CT339 from *C. trachomatis*. I ran BLAST analysis to identify the conserved competence motif then generated multiple sequence alignments and hydropathy predictions for structure. These indicated CT339 is a multi-pass transmembrane protein with structural homology to ComEC from *Bacillus*. I then used PCRs from three loci dispersed around the genomes of recombinant progeny to show that a disruption in CT339 prevents lateral gene transfer between *C. trachomatis* mutants and tetracycline-resistant *C. suis* R19. All recombinants with a *C. suis* OmpA seroreactivity incorporated the β -lactamase from *CT339::Tn*. No progeny showed tetracycline-resistance transfer into *CT339::Tn*. Experiments in which transformations were attempted with *CT339::Tn* mutants also showed no ability to uptake DNA, supporting the role of CT339 as both a structural and functional homolog of ComEC.

Together, these two approaches provide the ability to assess large genetic loci and single-gene roles in growth and infectivity of chlamydial species. Homologous recombination allows for generation of both gain- and loss-of-function mutants for the determination of genetic regions essential for pathogenesis; narrowing the genome for assessment of individual components within each region. Transposon mutagenesis offers the advantage of single-gene disruptions for direct genotype-phenotype correlations, as demonstrated by reduced bacterial burdens in mammalian infections and my discovery of CT339 as a homolog to ComEC.

ACKNOWLEDGEMENTS

Graduate school is the 8th layer of hell and I could not have crawled my scathing body from the rubble without the following individuals: First to my advisor Dr. P. Scott Hefty. You have always been an incredibly supportive mentor throughout every curveball life seemed to throw at me and your unwavering confidence in my abilities has been the foundation for my tenacity. Thank you for teaching me to drive my own train. To the members of the Hefty Lab, especially Katelyn, Megan, LaBrie, Bad Bob, and Aidan; you've all provided unending laughter and truly define the "Hefty Lab Family." LaBrie, you've always had my back. You're the most reliable person I've ever met. Aidan, "mah baby" you're going to be the most incredible physician scientist the world has ever seen and I am so incredibly grateful to have the opportunity to work with you. It is an honor to call you my friend and I cannot wait to see the immense impact you'll have on science and health. Bad Bob: thank you for all the encouraging words and pictures of your puppies. Onward and Upward! Katelyn, you've been here since my first day in Lawrence and Megan, you're truly my other half. The TRIFECTA has made these past few years bearable. I would also like to thank my committee, Drs. Hancock, Davido, Egan, Richter, and Rivera. Your guidance has been essential to the shaping of me as a scientist and all my future successes.

I would like to thank my friends and family, especially Nancy and the wallyball gang; Amy, Abby, and Morganne; and most importantly my parents and my sister. I apologize for the amount of anxiety I've put you all through and am so grateful for the love, support, and assurance you've had in me. Mom, dad, and Kim: you've taken the brunt of my complaints, freak-outs, and attempts at giving up, and encouraged me to continue. I love all of you so much and would never have achieved so much without you. Amy, Abby, and Morganne: You three

make my heart whole; you make me the person I want to be and push me to be my best. I cannot imagine life without you. I carry your heart in my heart.

I'd like to express my sincere thanks to Zach Howser and Lee Blankenship, without whom I would literally not be alive today; Dr. Corey Mayo for saving my leg (and my life), and my physical therapists Lisa, Leslie, and Adam, without whom I would not be able to walk.

Finally, I would like to thank Dr. Harry Kestler, to whom this work is dedicated. You have instilled my love for science and a passion for research, but most importantly, you have taught me that anything is possible with persistence. You have believed in me whole-heartedly, even after I moved on to graduate school, and you continue to be the driving force for every achievement in my life. You always had faith in me (and all your students), making us feel not just adequate, but extraordinary. Everything I have accomplished, both academically and personally, I attribute to your voice in my head, cheering me on. I can only hope to become half the mentor you've been and I cannot thank you enough. K-lab member for life.

This work is dedicated to Dr. Harry Kestler.
Persist like a Lentivirus.

TABLE OF CONTENTS

Chapter I. General Introduction

Chlamydial Impact on Public Health	1
Chlamydial Biology and Development	6
Advances in Genetic Systems for <i>Chlamydia</i>	11
Homologous Recombination	15
Horizontal Gene Transfer	18
Transposon Mutagenesis	20

Collaborator's Contributions	29
-------------------------------------	----

Chapter II. Lateral Gene Transfer Between *C. trachomatis* and *C. muridarum* Allows for Discovery of Genetic Loci Associated with Host-Adaptation and Infectivity

Abstract	30
Introduction	31
Materials and Methods	37
Results	42
Discussion	63

Chapter III. Single Insertion and Disruption Using a Novel Transposon Mutagenesis System for *Chlamydia* Identifies Genes Important for Mammalian Infection

Abstract	68
Introduction	69
Materials and Methods	74
Results	82
Discussion	108

Chapter IV. Genetic Disruption of the Hypothetical Protein CT339 Reveals an Essential Role in Lateral Gene Transfer in *Chlamydia*

Abstract	114
Introduction	115
Materials and Methods	118
Results	123
Discussion	139

Chapter V. Discussion

Advantages and disadvantages of homologous recombination as a means for identifying genetic correlates	143
Transformation efficiency is a limiting factor for library generation	147
Transposon mutants CT696 is highly specific to Chlamydial biology	150
Transposon mutants overcome deficiencies observed in recombination techniques	151
Identifying how CT339 enables DNA uptake	152

References

155

Chapter I. General Introduction

Chlamydial Impact on Public Health

As early as the 1900s, scientists have been investigating the “parasite” responsible for infectious blindness plaguing nearly 53 countries—later identified as the obligate intracellular bacterium, *Chlamydia* (Knirsch 2007, Nunes and Gomes 2014, Taylor, Burton et al. 2014). Over the next >100 years, research on numerous species, host adaptation, and basic biology would propel the organism to the forefront of microbial research and, most importantly, magnify the broad understanding of a radically atypical pathogen: one with a growing impact on public health and accumulating numbers of cases every year.

As of 2009, the genus *Chlamydia* consisted of the 9 major species capable of infecting a range of hosts including: *caviae* (guinea pigs), *felis* (cats), *pecorum* and *abortis* (mammals), and the more biologically relevant species: *suis*, a swine-adapted strain exhibiting antibiotic resistance capable of transmission into human-specific strains; *psittaci*, a species infecting birds with potential zoonosis into humans; *pneumonia*, a human-adapted strain causing lung infections and pneumonia; *muridarum*, a rodent strain ideal for research *in vivo* due to strikingly similar acute genital infections and post-infection sequelae mimicking those observed in humans; and *trachomatis*, the most prevalent species infecting humans (Longbottom and Coulter 2003, De Clercq, Kalmar et al. 2013, Nunes and Gomes 2014).

According to the Center for Disease Control and Prevention (CDC), over 1.5 million new cases of *C. trachomatis* infections occur annually, steadily increasing since 2000 (Figure 1.1). These numbers, however, do not include the other pathogenically relevant species such as *C. pneumonia*, which alone infects nearly 300,000 individuals in the United States annually; an

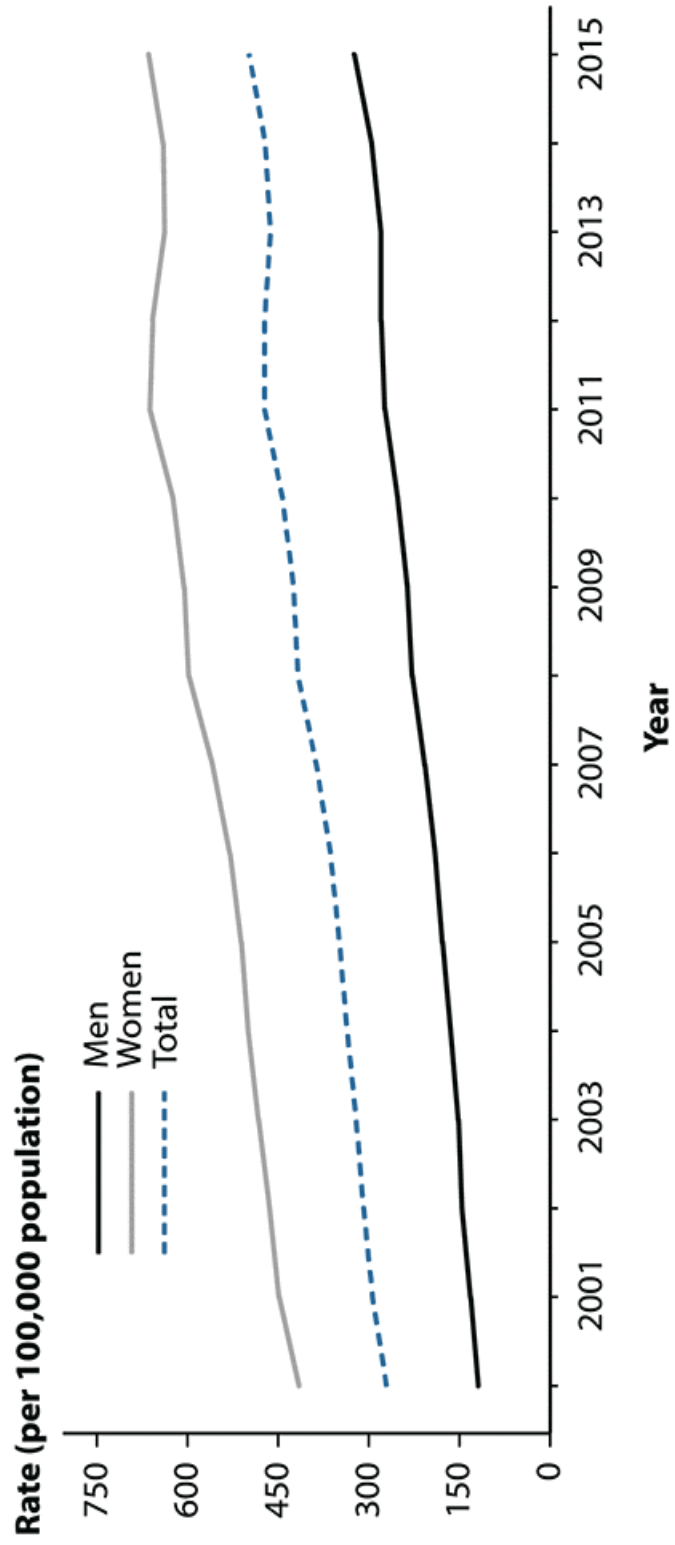


Figure 1.1 Rate of reported *C. trachomatis* infections by sex, United States, 2000-2015.

Figure acquired from the CDC 2015 Sexually Transmitted Disease Surveillance Report. It is noted that while data collection for *Chlamydia* infections began in 1984, it was not reportable in all 50 U.S. states and the District of Columbia until 2000. Increases in rates of reported cases may be due to both an increase in quality and awareness of testing capabilities and incidence of infection (U.S. Department of Health and Human Services).

approximation likely underestimated due to lack of reporting strategies and misdiagnoses (Cilloniz, Ewig et al. 2011).

Among the most prevalent *C. trachomatis* species, three distinct subclasses (serogroups) are recognized based on differences within the major outer membrane protein, OmpA, and dictate which tissue type the organism is most likely to infect. The first serogroup consists of organisms predominantly infecting the ocular epithelium (serovars A through C), and are the causative agent of endemic trachoma. Recent reports from the World Health Organization suggest about 1.2 million people within 51 countries where *Chlamydia*-induced trachoma is endemic are irreversibly blind. It is estimated that upwards of 40 million others are suffering from active trachoma infections and are at high risk for developing permanent scarring and future blindness. (Knirsch 2007, Wright, Turner et al. 2008, Ramadhani, Derrick et al. 2016).

Serovars D through K make up the second serogroup, infecting ano-urogenital tissue and collectively comprise the aforementioned 1.5 million sexually transmitted infection cases in the United States and estimated 90 million worldwide (Cochrane, Armitage et al. 2010, Tan and Bavoil 2012). These serovars are responsible for a number of reproduction-associated diseases including: proctitis and epididymitis in men, cervicitis and pelvic inflammatory disease in women, and urethritis and sterility in both sexes (Pellati, Mylonakis et al. 2008, Nunes and Gomes 2014).

The final serogroup causes genital tract infections termed lymphogranuloma venereum (LGV), and are primarily manifested through rectal penetration (Kapoor 2008, O'Byrne, MacPherson et al. 2016). Infections with these serovars (L1 through L3) are generally divided into 3 stages due to their ability to cause invasive and systemic infections through macrophage dissemination (Tan and Bavoil 2012, Nunes and Gomes 2014). Stage 1 presents with localized

inflammation, small lesions, and discharge within a month after exposure. The second stage of infection may result in flu-like symptoms such as fever, chills, and malaise, due to the diffusion of *Chlamydia* to the lymph; genitally, the infection remains asymptomatic. More severe infections result in mucosal ulcers, lymphangitis and suppurative necrosis forming buboes surrounding the inguinal ligaments (Tan and Bavoil 2012, O'Byrne, MacPherson et al. 2016). Finally, the third stage of infection results in irreversible damage, sterility due to tissue scarring, and genital elephantiasis (Dal Conte, Mistrangelo et al. 2014, O'Byrne, MacPherson et al. 2016). Until recently, LGV infections in the developed world and western hemisphere were rare. Since 2003, outbreaks have been identified in the Netherlands, U.S., U.K., and Australia, with the majority of cases occurring within men who have sex with men (Kapoor 2008, O'Byrne, MacPherson et al. 2016).

Notwithstanding the high reports of infections, there is almost no evidence of antibiotic resistance in human-adapted *Chlamydia* species (Sandoz and Rockey 2010). *C. suis*, the swine-adapted species, has shown acquisition of a genomic island containing tetracycline resistance genes; however, this is probably due to the high rate of antibiotic use as a means of growth promotion, driving the selection of rare organisms that have undergone this acquisition (Nunes and Gomes 2014). Indeed, clinical trials with two broad-spectrum antibiotics, azithromycin and tetracycline, resulted in over 95% of bacterial clearance and over 92% of long-term effectiveness (Lau and Qureshi 2002, Golden, Whittington et al. 2005, Geisler, Uniyal et al. 2015). Although treatment failures have been clinically reported (Batteiger, Tu et al. 2010, Geisler, Uniyal et al. 2015), meta-analysis reports that the majority of lasting infections are the result of reinfections as opposed to antibiotic failure. Further, stressors such as beta-lactam antibiotics, as well as host immune responses, may induce a persistent state in many chlamydial species, including *C.*

trachomatis (Beatty, Morrison et al. 1994). This state results in metabolically inactive, “aberrant” forms of the organism, which are capable of evading treatment strategies. Upon removal of the stressor, either antibiotic clearance or the immune response diminishing, aberrant *Chlamydia* revert back to the active infectious cycle, resulting in chronic infections (Schoborg 2011). It is because of this phenotype, along with the majority of *Chlamydia* infections remaining asymptomatic that result in the ever accruing WHO and CDC disease reports and the necessity for more efficacious prophylactic approaches. To date, there is no vaccine available for any *Chlamydia* species, however, this is not due to lack of effort; simply, many facets of the organism’s biology, including correlates of immunity, remain poorly understood (Nunes and Gomes 2014, Rey-Ladino, Ross et al. 2014).

Chlamydial Biology and Development

As mentioned above, the majority of complications in designing pathogen-specific antibiotics, inhibitory pharmaceuticals, or vaccines against chlamydial species are due to a general ignorance of the basic biology of the organism. *Chlamydia* species have a highly conserved ~1Mb genome and ~7.5kb plasmid, with as many as one-third of the coding regions resulting in proteins of unknown function, more commonly termed “hypothetical proteins” (Stephens, Kalman et al. 1998, Nunes, Borrego et al. 2013). Indeed, this lack of annotation has remained the crux of the biological setbacks for the organism and, given a complicated lifestyle yet reduced genome, there remains a number of cellular processes these unknowns may be playing a part in; with high probability for multifactorial roles and compensatory functions.

All species of *Chlamydia* are incapable of growth and survival outside of a host regardless of tissue tropism. They all maintain a complicated biphasic development cycle with two distinct forms known as the elementary body (EB) and reticulate bodies (RBs) (Abdelrahman and Belland 2005). The infectious cycle begins in which the metabolically inactive EBs bind to a host cell and initiate entry (Figure 1.2). A number of factors play a role in the attachment and entry of EBs into the host cells. This includes adhesion proteins such as the outer membrane proteins OmpA, OmcB and Hsp70, polymorphic membrane proteins (Pmps), and proteins involved in the type III secretion system (TTSS), such as the translocated actin recruiting phosphoprotein (TARP) (Abdelrahman and Belland 2005, Betts, Wolf et al. 2009). TARP functions to modulate host cell actin for the rearrangement and engulfment of *C. trachomatis*, yet it has been shown to be dispensable with EBs remaining capable of entry and infection when TARP is mutated or treated with inhibitors (Betts, Wolf et al. 2009, Parrett, Lenoci et al. 2016). Combined, these proteins aid in what was initially classified as ‘parasite-specified phagocytosis’ in which EBs are shown to induce uptake within the host cell. However, many contradictory reports have demonstrated both the necessity and lack thereof for key receptors including, dynamin, clathrin, and lipid rafts (Dautry-Varsat, Subtil et al. 2005).

Once internalized, chlamydial EBs modify the parasitophorous vacuole, termed an inclusion, to evade the lysosomal-fusion pathway and manipulate the environment to promote growth and replication (Fields and Hackstadt 2002). As early as 2 hours post-infection, chlamydial EBs begin to undergo differentiation into the metabolically active, noninfectious RB form via unknown signals (Abdelrahman and Belland 2005, Tan and Bavoil 2012). Once differentiated, RBs localize to the edges of the inclusion for protein secretion across the membrane, including deubiquitinases (Le Negrate, Krieg et al. 2008), apoptosis inhibitors

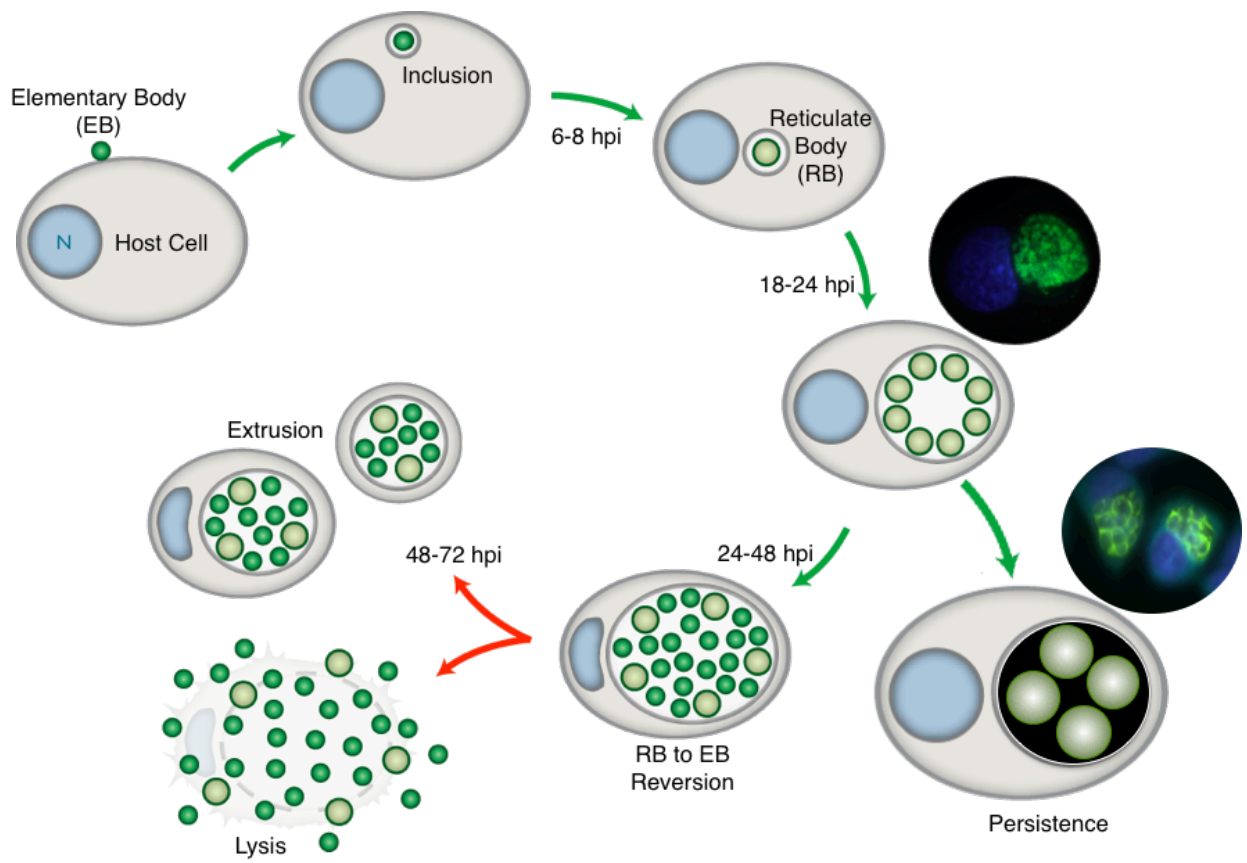


Figure 1.2 *Chlamydia* developmental cycle. The infectious elementary body (EB) of *Chlamydia* first attaches and enters the host cell through a modified endocytosis. After roughly 8 hrs, EBs convert to their metabolically active reticulate bodies (RBs) where they grow and divide. Around 24-48 hpi, RBs begin reverting back to EBs and finally around 72 hours, *Chlamydia* either lyses the host cell or exits through a process known as extrusion, infecting neighboring cells. Under stressful conditions including nutrient starvation or the presence of antibiotics, *Chlamydia* enters a persistent state. Persistence results in large aberrant forms incapable of dividing or reverting back to EBs. Upon removal of the stressor, the aberrant forms revert back to RBs and continue on the normal developmental process. Figure modified from Kevin Hybiske. Insert—Green: MOMP staining of *Chlamydia* RBs, Blue: DAPI stain of host cell nucleus.

(Fan, Lu et al. 1998), and proteins involved in nutrient acquisition (McClarty 1994). During this time, RBs are rapidly dividing through binary fission, generating over 1,000 organisms per infected cell (Tan and Bavoil 2012). Roughly 24-48 hours post-infection, RBs begin reversion back into EBs; this phase is considered asynchronous in that, while some RBs terminally differentiate back into the infectious form, many remain actively dividing to produce more RBs (Tan and Bavoil 2012). This hallmark of development illuminates another step in which cellular signals remain poorly understood. It has been speculated that RB dissociation from the membrane or TTSS removal may induce a reversion signal (Bavoil, Hsia et al. 2000). Finally, roughly 48 hours after initial infection, most RBs have reverted back into EBs and are released from the host cell using one of two methods: extrusion (the pinching off of the chlamydial inclusion enveloped in host-membrane), or lysis of the host cell (Hybiske and Stephens 2007, Zuck, Sherrid et al. 2016).

As mentioned previously, in order to evade the host immune responses, as well as in the event of nutrient starvation or the presence of certain types of antibiotics, *Chlamydia* are capable of entering a persistent state (Figure 1.2). During this state, RBs expand, converting into an aberrant form (Schoborg 2011). A number of triggers for inducing these forms have been identified, including tryptophan starvation through host IFN-gamma induction, nutrient deprivation including amino acids and glucose, as well as the presence of penicillin, ofloxacin, or ciprofloxacin (Segreti, Kapell et al. 1992, Dreses-Werringloer, Padubrin et al. 2000). Upon removal of the antibiotics or reintroduction of necessary nutrients, *Chlamydia* reverts back to normal growth; however, studies have shown *C. trachomatis* is capable of resistance to azithromycin when previously exposed to penicillin antibiotics (Wyrick and Knight 2004). Tryptophan depletion due to IFN-gamma activity is the result of indoleamine 2,3-dioxygenase

(IDO) activation (Bonner, Byrne et al. 2014). IDO activation initiates the degradation of intracellular tryptophan levels, resulting in reduced levels of the amino acid available for chlamydial protein synthesis. Conversely, *C. trachomatis* possesses a tryptophan synthetase capable of converting indole produced by normal microflora in the genital tract into usable tryptophan (Xie, Bonner et al. 2002, Nelson, Virok et al. 2005, Bonner, Byrne et al. 2014). Through both the presence of aberrant forms, and overcoming host-tryptophan depletion, persistent infections occur producing sustained inflammation and tissue damage, resulting in the long-term sequelae alongside high frequencies of infection.

Advances in Genetic Systems for *Chlamydia*

Due to the obligate intracellular nature of *Chlamydia*, along with the biphasic lifestyle, incorporation of foreign DNA (e.g. plasmid) has been problematic. EBs are capable of survival and infectivity extracellularly and as such, provide an anchor for exogenous DNA uptake into the host cell and inclusion; however EBs contain a rigid cell wall of tightly cross-linked proteins, prohibiting DNA from entering. On the other hand, the replicative RB form does not have the rigid cell wall, rendering it more susceptible to DNA acquisition. Unfortunately, RBs reside both within the host cell and inclusion, thus DNA is required to pass through four distinct membranes without degradation—host membrane, inclusion membrane, and RB inner and outer membranes (Bastidas and Valdivia 2016). To overcome this, external attachment or coupling of DNA to the EB surface is one method for integration of plasmid vectors but presents the challenges of environmental degradation, detachment, or disruption of essential EB/host interfaces..

It was only within the past few decades that foreign DNA has been transiently introduced to *Chlamydia*; initially through electroporation and antibiotic selection using chloramphenicol resistance (Tam, Davis et al. 1994). It was not until nearly 14 years later that this system would be improved upon for the development of stable transformants in *C. psittaci* (Binet and Maurelli 2009). It was found through these studies that exceedingly high concentrations of plasmid DNA was required, ranging from 5 to 20 μg , and resulted in reasonably low frequencies of transformation, identifiable only after several rounds of serial passages (Bastidas and Valdivia 2016). In order to isolate clonal populations of these transformants, chlamydiologists developed modified plaque assays, adapted from those designed for viral particle counts (Matsumoto, Izutsu et al. 1998). However, clinical isolates, as well as environmental strains of *Chlamydia*, have shown to plaque poorly (Bastidas and Valdivia 2016).

Chemical transformation with shuttle plasmids designed for use in chlamydial spp. provided the next leap forward for introducing plasmid DNA into the organism, but, again, this represented a complicated and relatively ineffective method. Recombinant plasmids are often not taken up in the presence of a native plasmid and, in most cases of plasmid acquisition, native plasmids replicate more efficiently, resulting in deletion of the foreign plasmid and loss of gene transformation (Nordstrom and Austin 1989). Given that *Chlamydia* contain a native plasmid; it became imperative for transformation that plasmid-free isolates of *C. trachomatis* be developed. Wang et al. were incapable of curing LGV of its native plasmid and as a result, designed a shuttle vector system in which the chlamydial native plasmid was fused with their genes of interest, eliminating the complications associated with a recombinant plasmid (Wang, Kahane et al. 2011). This shuttle vector incorporated both chloramphenicol and penicillin resistance genes, providing a simple method for isolating transformed clones using selection with either antibiotic.

Standard laboratory protocols for transformation of bacteria calls for incubation with CaCl_2 followed by a “heat shock” to facilitate the entry of the plasmid DNA (Mandel and Higa 1970). It was found that, upon incubation of EBs with CaCl_2 and McCoy cells (the standard fibroblast cell line used for growing *Chlamydia*), transformation was possible and heat shock was unnecessary. Recovery of bacteria was allowed to occur for at least one complete round of the developmental cycle before penicillin selection was implemented (Wang, Kahane et al. 2011). Stably transformed *C. trachomatis* was observed after three rounds of selection and transformation was verified via Southern Blotting. The development of a shuttle vector by Wang et al. marked an essential development in the field of chlamydial genetics. It allowed for the potential introduction of genes and use of genetic systems for the elucidation of previously unknown properties of chlamydial biology.

In 2013, Wickstrum et al. designed a new shuttle vector utilizing a tetracycline-inducible system for the expression or repression of genes (Wickstrum, Sammons et al. 2013). In this system, the tetracycline repressor, TetR, remains tightly bound to operators found within the *tetA* promoter: *tetO*₁ and *tetO*₂. When bound, TetR strongly represses transcription of genes under the control of the *tetA* promoter. The presence of tetracycline or tetracycline derivatives then reduces the binding of the repressor to the operators, allowing for transcription of the downstream gene(s) of interest (Bertram and Hillen 2008). Fusing the tetracycline-inducible system to the native chlamydial plasmid, Wickstrum et al., were able to tightly control the expression of GFP under the control of tet-induction (Wickstrum, Sammons et al. 2013). Thus began the steeplechase to identify gene functions within *Chlamydia*.

In addition to revolutionizing the capacity for exogenous DNA uptake, advances in chlamydial recombination have propelled the field into a new era of genetic discovery. Whole

genome sequencing of *C. trachomatis* showed that DNA recombination systems are “extensively represented” and therefore could be exploited for phenotype analyses (Stephens, Kalman et al. 1998). DeMars et al. showed that antibiotic resistance acquisition via recombination occurred roughly 10^4 times more frequently than resistance due to spontaneous mutations and confirmed the genetic stability of *in vitro* recombinants (Demars, Weinfurter et al. 2007). Through recombination, a number of chimeras with the potential for innovative characteristics can be developed and the particular genetic loci associated with these phenotypes assessed. Indeed the observation of tetracycline resistance within *C. suis* and subsequent transfer to *C. trachomatis in vitro* shows the potential for cross-species recombination in order to identify genetic correlates, potentially hypothetical proteins, associated with host- and tissue-tropisms. Further, the transfer of antibiotic resistance genes into human-adapted *Chlamydia* strains reveals the foreboding public health obstacles looming on the horizon.

Modern techniques for genetic manipulation have also been developed for use in *Chlamydia* including targeted mutagenesis with the TargeTron system (Johnson and Fisher 2013), chemical mutagenesis using ethyl methanesulfonate (Nguyen and Valdivia 2014), and gene deletions via allelic exchange (Mueller, Wolf et al. 2016). While each of these systems has benefits, many of them have disadvantages as well. TargeTron systems are dependent on knowing which gene to target and, with over 300 hypothetical gene products, it is difficult to know where to begin. Further, this system has demonstrated to be relatively inefficient in *Chlamydia* (Bastidas and Valdivia 2016). Chemical mutagenesis allows for spontaneous mutations to occur, overcoming the issue of having to select specific genetic targets for disruption. However, the numbers of mutations that occur eliminate the ability to associate a specific phenotype with a single gene disruption, requiring a follow-up application of targeted

genetics (Kari, Goheen et al. 2011). Allelic exchange combines both the groundbreaking ability to transform *Chlamydia*, with the ability to target specific endogenous genes for deletion. This system further allows for the introduction of new genes as well as the complementation of mutated or knockout genes, affording the ability to complete the molecular Koch's postulates (Mueller, Wolf et al. 2016). Despite the robust advances made in genetic manipulation of *Chlamydia*, each method results in varying degrees of efficiency and brings variable levels of shortcomings. Future efforts to overcome these inadequacies are essential for the advancement of chlamydial biology, identification of gene products and functions, and discovery of innovative biomarkers for pharmaceutical developments.

Homologous Recombination

The process of homologous recombination is both well defined and highly utilized as a tool for gene mutation in pro- and eukaryotes (Packer and Liu 2015). While discoveries in microorganisms came later than those identified in higher-order organisms, it would become one of the most well-characterized pathways in bacteria (Michel and Leach 2012).

The central protein within the recombination pathway and the first discovered in *E. coli* was RecA. With its crystallization in 1992, a number of domains essential for function, including two proposed DNA binding domains, were confirmed (Story, Weber et al. 1992, Michel and Leach 2012). These two domains are hypothesized to bind single-stranded DNA to double-stranded DNA with complementary sequences, resulting in strand invasion and DNA synapsis reactions. Accessory proteins RecB, C and D initiate recombination through unwinding and “chewing back” of donor DNA. Almost all foreign DNA taken up by bacteria, either through

conjugation, horizontal gene transfer, or bacteriophage injection, undergoes this process (Lawrence and Retchless 2009). Upon recognition of a specific sequence known as the Chi site, RecA is recruited and strand invasion occurs, displacing the second strand from the resident, receiver DNA, forming what is called a D-loop. This D-loop can either be recognized as a replication fork, resulting in the generation of only one strand of recombinant DNA, or it can be nicked, resulting in a Holliday junction. Holliday junctions are further resolved using a DNA repair system RuvABC or RecG and result in two recombinant DNA strands (Michel and Leach 2012). As mentioned previously, components from these systems have been extensively researched in *E. coli* for their roles in regulation and assistance in recombination; RecB and C proteins have been shown to have recombination deficient effects if mutagenized, however inactivation of RecD results in multimerization of plasmids and hyper-recombination events (McGrew and Knight 2003, Michel and Leach 2012).

Within many bacteria, homologous recombination is an essential component for the evolution and “shuffling” of DNA—allowing for acquisition and/or removal of genes which may be either beneficial or damaging (Packer and Liu 2015). Recent studies in a number of organisms have shown the ability to identify novel virulence factors acquired through homologous recombination. Clinical isolates of *Neisseria meningitides*, for example, have highly dynamic genomes, classified by what is known as “sequence types.” Through genome analysis of two invasive strains within the same sequence type, ~35 “non-vertically” transmitted genes originating from different sequence types have been identified, contributing to the pathogenicity of the organism (Kong, Ma et al. 2013). Structural differences due to unequal recombination and duplication events within virulence factor *cagA* from *Helicobacter pylori* has led to emergence of geographically diverse elevations in virulence and chimeric C-terminal motifs (Furuta, Yahara

et al. 2011). Further, homologous recombination has been exploited experimentally for direct mutagenesis of organisms and analysis of gene roles in pathogenicity, as in *Rickettsia prowazekii*. Recombination resulted in replacement of WT phospholipase D with a partially deleted version, demonstrating the functionality of this gene product in bacterial survival (Driskell, Yu et al. 2009).

In *Chlamydia*, homologous recombination was previously assumed improbable due to its intracellular nature and lack of infections with more than one strain (Harris, Clarke et al. 2012), however genome sequencing of *C. trachomatis* suggested otherwise. Variability within the outer membrane proteins (particularly *ompA*) and polymorphic membrane proteins (*pmps*) is observed, leading researchers to investigate whether key players in homologous recombination are present in *Chlamydia*, particularly the central player: RecA. Genome fragments from *C. trachomatis* were cloned and transformed into a RecA knockout *E. coli* strain and screened for a rescue in recombination. Coincidentally, the *E. coli* transformant exhibiting rescue consisted of a chlamydial fragment containing only one major open reading frame which, when compared to sequences of known RecA proteins, showed identities above 50% (Hintz, Ennis et al. 1995, Zhang, Fan et al. 1995).

From these discoveries, the analyses of many chlamydial genomes identified numerous historical recombination events. Joseph et al. identified over 300 recombination events within the genomes from tissue isolates of *C. suis*, as well as DNA from both *C. trachomatis* and *C. suis* present within the *C. muridarum* genome, although they were not recombined among themselves (Joseph, Marti et al. 2016). Computationally, it is estimated that recombination affects short nucleotide segments, averaging ~350 bp with the majority occurring in serogroup two (serovars D through K), and the least amount of recombination occurring in LGV specific isolates (Joseph,

Didelot et al. 2012). One hypothesis for this is the environmental impacts placed on recombinant strains (Lawrence and Retchless 2009). Whereas serovars from group two may be exposed to each other quite frequently within the same environment through repeated infection and exposure, LGV-specific serotypes rarely co-culture *in vivo* with serovars outside this specific clade. Similarly, it is likely that regions surrounding niche-specific genes show lower rates of homologous recombination naturally; a theory assessed within later chapters of this dissertation.

Horizontal Gene Transfer

One very important mistake often made by geneticists involves differentiation between homologous recombination and horizontal gene transfer. Horizontal gene transfer typically refers to the method of DNA acquisition as well as potential origins, however the incorporation of new DNA into a recipient genome requires the ability of homologous recombination and thus, the two should be considered concurrently.

Within *Chlamydia*, the first and most obvious example of horizontal gene transfer (HGT) is the presence of the genes encoding ATP/ADP transport proteins, allowing intracellular organisms to exchange ADP with host-manufactured ATP (Tjaden, Winkler et al. 1999). Evidence indicates that an ancient chlamydial ancestor, living as an endosymbiont, acquired these genes horizontally, potentially after a series of gene duplication events. Transport proteins such as these have historically only been utilized by plant and algal chloroplasts. In fact, *Chlamydia* genomes assessed for horizontal transfer have identified a greater number of genes gained from plants than those from metazoans and fungi (Koonin, Makarova et al. 2001). Recent follow-up studies have further identified similarly functional transport proteins in

Acanthamoeba, which is the recently classified *C. trachomatis*-related bacteria (Schmitz-Esser, Linka et al. 2004).

Along with acquisition of single genes, the ability for entire operons or islands to transfer into new bacteria has been confirmed in a number of bacterial genomes, including *Chlamydia*. Koonin et al. identified operons containing Na⁺-transporting NADH:ubiquinone oxidoreductase and archaeal/vacuolar-type H⁺-ATPases horizontally transferred into *C. trachomatis* and *C. pneumoniae* (Koonin, Makarova et al. 2001) and the presence of a tetracycline resistance genomic island within *C. suis* isolates has been widely acknowledged (Dugan, Rockey et al. 2004).

One key process for HGT is the ability of bacteria to transform, or take up exogenous DNA. This process requires a number of proteins involved in DNA binding and processing and ultimately converges with the recombination pathway described above. Whereas some components of the system remain highly conserved between Gram-positive and Gram-negative bacteria, the presence of both an outer- and inner-membrane in Gram-negative bacteria necessitates additional protein constituents. In Gram-positive bacteria lacking an outer membrane, double-stranded “donor” DNA is bound by ComGC, a pilus extending beyond the peptidoglycan layer, and guided to the receptor protein ComEA. It is then degraded into single-stranded DNA by the EndA nuclease (Dubnau 1999, Berge, Moscoso et al. 2002, Chen and Dubnau 2004, Johnston, Martin et al. 2014). This single-stranded DNA is then internalized through the ComEC transmembrane channel via the ComFA ATP-dependent translocase protein (Dubnau 1999). Once internalized, various processing proteins bind the single-stranded DNA, leading to the recruitment of RecA and progressing to homologous recombination (Johnston, Martin et al. 2014).

Gram-negative bacteria, on the other hand, utilize the type IV pilin/secretin channel for transport of DNA across the outer membrane (Figure 1.3). DNA is bound by the PilE subunits making up the pilus and is transported through the outer membrane via transmembrane secretin, PilQ (Chen and Dubnau 2004). Within the periplasm, the homolog of ComEA, ComE, binds DNA and delivers it through the inner membrane channel, ComA. While it is known that DNA must be processed into single-stranded DNA for recombination, the presence of a homolog to ComFA (the translocase protein in Gram-positives) has not been confirmed, nor has any nuclease similar to EndA been identified (Chen and Dubnau 2004). In *C. trachomatis*, while there is evidence for horizontal gene transfer, none of the bacterial uptake proteins described above have been annotated.

Transposon (Tn) Mutagenesis

Arguably one of the most powerful tools for the study of pathogenesis has been the optimization of transposon mutagenesis. The ability to design any gene insert as the transposon itself, coupled to its applicability to a vast number of organisms, makes it one of the most groundbreaking tools available for genome manipulations. Since their discovery over fifty years ago in *Zea mays* by Barbara McClintock, “jumping genes” have provided a number of options for researcher. These include the ability to assess phenotypes from disrupted genes, the potential for gain-of-function mutants through the insertion of new open reading frames, and transposon

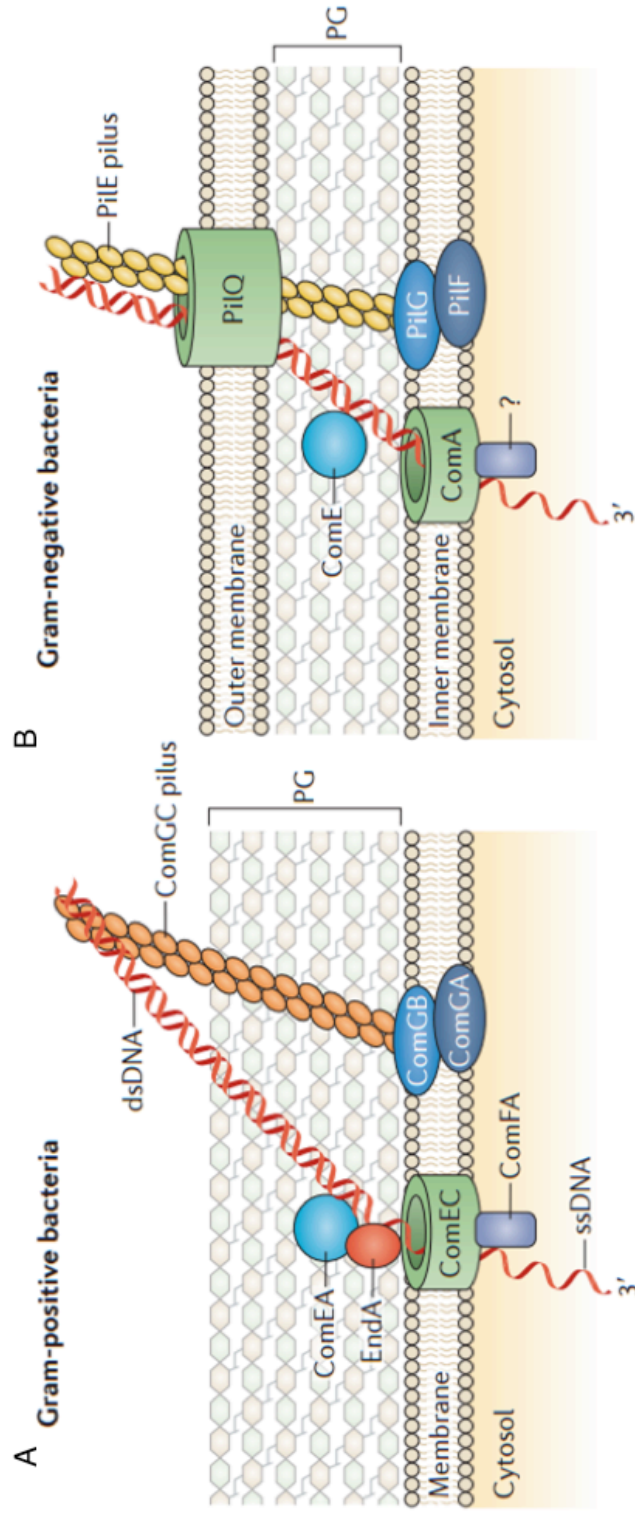


Figure 1.3 Scheme of Bacterial DNA uptake machineries. A) In Gram-positive bacteria, ComGC subunits make up the transformation pilus, which captures double-stranded DNA (dsDNA) and brings it to the DNA receptor ComEA and transmembrane pore ComEC. The EndA nuclease breaks the dsDNA into single-stranded DNA, which is then brought into the cell through the ATP-dependent translocase ComFA. ComGB and ComGA stabilize the ComGC pilus. B) In Gram-negative bacteria, the transformation pilus is composed of Pile subunits and dsDNA is internalized through the outer membrane via PilQ. The DNA receptor homolog ComE guides DNA in through the ComEC transmembrane pore homolog, ComA. The EndA nuclease homolog remains unknown. PilG and PilF stabilize the Pile pilus within the inner membrane. PG=Peptidoglycan.

Adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Microbiology] (Johnston, C., B. Martin, G. Fichant, P. Polard and J.-P. Claverys (2014). "Bacterial transformation: distribution, shared mechanisms and divergent control." *Nat Rev Micro* **12**(3): 181-196), copyright (2014).

sequencing—the utilization of whole-genome sequencing combined with transposon mutagenesis to identify genotype/phenotype correlations on a genome-wide scale.

Two major classes of transposable elements (TE) have been augmented for use in research. Class I elements require the reverse-transcription from an RNA intermediate (Finnegan 1992, Hamer, DeZwaan et al. 2001). This class of TE has been widely used in mutagenesis of eukaryotes and is the main class of TE found within viruses, containing two subclasses, I.1 and I.2 (Finnegan 1992, Gebert and Rosenkranz 2015). Both subclasses contain open reading frames similar to the retroviral *gag* and *pol* genes, in that, they code long polyproteins. Class I.1 elements also contain long terminal repeats, whereas Class I.2 elements contain A-rich sequences at the 3' tails (Finnegan 1992, Konnyu, Sadiq et al. 2013).

The mechanisms for how Class I TEs integrate into the genomes of respective organisms also mimic that of retroviral infections; transcription of the TE depends on one long terminal repeat for initiation, typically at the 5' end, and contains open reading frames for the reverse transcriptase. Upon transcription completion, promoter sequences for the reverse transcriptase allow for translation of the enzyme that then reverse transcribes the remaining RNA construct into double-stranded DNA (Deininger and Batzer 2002). For Class I.1 TE, this double stranded DNA is then incorporated at specific sequences within the genome using nicks—a method similar to Class II TE (see below). For Class I.2 TE, the mechanism for DNA integration remains poorly understood. Contradictory research results show that gene rearrangement and truncations may occur resulting in point mutations often associated with this class of TEs and is highly dependent on cellular enzymes for nicks, ligation, and other processes. Regardless of subclass, Class I TEs are always replicative, meaning that the original RNA transcript is never removed.

Because of this, accumulation of these insertions may occur over many generations (Deininger and Batzer 2002).

Class II TEs are undoubtedly the more widely used class for bacteria due to the ease of application. These TEs transpose directly from DNA into DNA using their own internal transposase, removing the necessity for reverse transcription and genetic modification within the cell (Hamer, DeZwaan et al. 2001). These mechanisms require only magnesium, GTP and the respective insertion site (sequence) within the genome of the receiving organism and result in a non-replicative insertion. These TEs also appear less frequently within human and plant genomes (Finnegan 1992, Ammar, Izsvak et al. 2012). One noteworthy example of Class II TEs within the human genome are the RAG1 and RAG2 genes required for VDJ-joining in antibody development. It is estimated that these proteins have existed among jawed vertebrates for nearly 500 million years, transposed within the human genome, conferring an evolutionary advantage and thus remaining conserved (Kapitonov and Jurka 2005).

Given the ease of employment, Class II TEs have become essential tools for molecular biologists. The two most prevalent systems for DNA transposons include the *PiggyBac* (*PB*) system and *Sleeping Beauty* (*SB*). The newly designed *PB* system functions by the standard “cut and paste” mechanism common within Class II TEs, but only inserts at TTAA regions in a genome (Di Matteo, Matrai et al. 2012). Compared with viral vectors for genetic insertions, *PB* is more convenient to generate and results in more precise insertion due its lack of the requisite DNA synthesis for sealing gaps (Yusa 2015, Zhao, Jiang et al. 2016). In contrast to other Tn systems, *PB* has a number of advantages: anywhere from 9 to 14 kb-long fragments can be used, typically integrating as single-copy transgenes, with higher efficiencies of transposition, offering

precise excision and restoration of wild type sequences. They can be readily located using traditional molecular methods (Zhao, Jiang et al. 2016).

Notwithstanding the benefits of the *PB* system, two major disadvantages remain: 1) *PB* generally requires that the transgene and transposase be present on two separate plasmids to account for their increased sizes, requiring dual-plasmid uptake, and 2) *PB* was derived from insects, therefore codon usage within bacteria and mammalian cell lines remains an issue, resulting in a decrease in expression. While codon-optimization and mutating inverted repeat sequences has resulted in dramatic increases in both excision and integration within a genome, the issue of dual plasmid uptake remains (Di Matteo, Matrai et al. 2012). An attempt to combine the transposon and transposase on one vector, resulted in irregular integration, weak transposase expression, and variable levels of GFP transgene expression (Chakraborty, Ji et al. 2014). These initial attempts at modification, however, highlight the deficiencies within the system and pave the way for improvements.

Sleeping beauty is so named due to its “awakening” or resurrection from the *Tc1/mariner* element found in fish genomes—these TEs were originally thought to be nonfunctional in vertebrates due to their accumulation of mutations. They have since been engineered for functionality; in fact, this system represents the first-ever ancient gene to be actively reconstructed (Ivics and Izsvak 2015). Using highly conserved consensus regions within the *Tc1/mariner* family, *Sleeping Beauty* was engineered, establishing the overall structure (the DNA-binding domain and the catalytic domain) now found in all *mariner* superfamily transposases (Plasterk, Izsvak et al. 1999). Similarly, the mechanism for transposition is also retained in the superfamily, broken into 4 major steps: 1) binding of the transposase to the inverted repeats, 2) pairing of the homologous IRs, 3) excision of the donor site, and 4)

integration into the target site. One of the major benefits to the *SB* system is its integration site selection—almost exclusively between AT nucleotides (Ivics and Izsvak 2015). The opportunity for randomized insertion across a genome provides significant benefits scientifically, however, it has been shown that palindromic AT regions with potential for hydrogen bonding generates genome “hotspots,” or preferential regions of insertion. Not surprisingly, this structural predilection is conserved among other *Tc1/mariner* transposon systems.

Similar to the *Sleeping Beauty* system, another *mariner* transposon called *Himar1* has been shown to have stable integration of transgenes and, upon mutation, has shown an increased frequency of transposition as compared to WT *Himar1* (Lampe, Akerley et al. 1999, Keravala, Liu et al. 2006). This “C9 hyperactive transposase” has been extensively applied for analysis of pathogenesis in a number of organisms including intracellular bacteria (Maier, Casey et al. 2007, Beare, Howe et al. 2009, Cheng, Nair et al. 2013). The C9 hyperactive transposase also inserts randomly between AT nucleotides, however, the propensity for long palindromic sequences and easily hydrogen-bonded regions, as is observed in *SB*, have not been assessed.

Regardless of system, transposon mutagenesis as a whole has provided inestimable insight into the pathogenesis of a number of bacteria. Genome-wide, random insertions have identified genes responsible for abscess formation from *Streptococcus intermedius* (Hasegawa, Sekizuka et al. 2017), hyper-invasiveness of *Campylobacter jejuni* (Javed, Grant et al. 2010), and biofilm formation in uropathogenic *E. coli* (Hadjifrangiskou, Gu et al. 2012). To date, however, the development of a transposon mutagenesis system functional in *Chlamydia* has not been described. Thus, the research described herein defines a functional Tn system within *C. trachomatis* and proceeds to characterize a small library of Tn mutants.

The central theme of this research focuses on identifying genomic components responsible for varying degrees of pathogenicity. For each area described above, a unique application resulted in appreciable information including identifying multi-gene loci responsible for specific phenotypes and potential gain-of-function mutants (horizontal gene transfer and homologous recombination). Equally, this also includes single gene disruptions through transposon mutagenesis for loss-of-function phenotypic analysis. Combined, this research has identified 1) a disinclination for *C. trachomatis* to acquire cytotoxin genes when allowed to recombine with *C. muridarum in vitro*, 2) mutants of FAD-dependent monooxygenase and ChlaDub1 from *C. trachomatis*, and MACPF, *glgB* and glucose-6-phosphate isomerase from *C. muridarum* show a decrease in recoverable organisms *in vivo* using a mouse-infection model and 3) a critical role for CT339 of *C. trachomatis* in lateral gene transfer. Chapter II describes my characterization of *C. trachomatis* and *C. muridarum* recombinant progeny generated by Bob Suchland at the University of Washington. I used PCR and sequence analysis to determine regions of genetic crossover and a comprehensive evaluation of genes gained and lost through recombination. With the help of Nancy Schwarting, I then used the mouse vaginal model of infection to determine if hybrids containing two of the *C. muridarum* cytotoxins were capable of ascension to the upper genital tract. I used droplet digital PCR on both ovaries and uterine horns to determine bacterial burdens in each organ.

Chapters III and IV describe the use of transposon mutagenesis systems developed by Jason Wickstrum and Dr. Scott Hefty to generate single-gene disruptions in *C. trachomatis* and *C. muridarum*. *C. trachomatis* Tn mutants were generated by Jason, Michael Barta and Gregory Peterson. Scott LaBrie did microscopic analysis, and Greg Peterson performed flanking PCRs and southern blots to identify single inserts in *C. trachomatis* mutations. *C. muridarum* Tn

mutants were generated by Yibing Wang and Kevin Hybiske at the University of Washington. I ran droplet digital PCR to identify multiple insertions in *C. muridarum* Tn mutants. I propagated mutants from both libraries and assessed bacterial burdens *in vivo* with the assistance of Nancy Schwarting. Chapter IV focuses on the role of CT339 in lateral gene transfer. I performed the *in silico* analysis and generated the multiple sequence alignments, hydropathy plots and topology predictions. Bob Suchland generated recombinant clones between *CT339::Tn*, *CT383/4::Tn* (IGR) and *C. suis* R19. I performed PCRs on loci dispersed across the genomes to identify parental backbone and directionality of genetic transfer. Chapters III and IV were combined and modified to include only the *C. trachomatis* Tn characterizations and submitted to mBio. This manuscript is currently under review.

Collaborator's Contributions

Bob Suchland at the University of Washington generated all recombinant clones used in this research. Brian Brunelle at the USDA, , performed whole genome sequencing of recombinant hybrids and generated contigs for sequence analysis. Jason Wickstrum and P. Scott Hefty generated plasmids used for transposon mutagenesis. *C. trachomatis* libraries of mutants were generated by Jason Wickstrum, Michael Barta, and Gregory Peterson. Gregory Peterson also performed flanking PCR, Southern blotting, and ddPCR of *C. trachomatis* Tn mutants. *C. muridarum* Tn mutants were generated by Yibing Wang and Kevin Hybiske at the University of Washington. Scott LaBrie, with assistance from the Microscopy and Analytical Imaging core at the University of Kansas, performed all microscopy and generation of Figures 3.6 and 3.7. Nancy Schwarting assisted in all animal experiments.

Chapter II.

Lateral Gene Transfer Between *C. trachomatis* and *C. muridarum* Allows for Discovery of Genetic Loci Associated with Host-Adaptation and Infectivity

Abstract

Chlamydial infections are the most commonly reported bacterial STI and the leading cause of preventable blindness worldwide. Notwithstanding this impact on public health, chlamydial pathogenesis remains poorly understood. One feature of *Chlamydia* biology is the variable host specificity and infectability despite highly similar genomic content between strains. An essential property of infection differential between murine (*C. muridarum*) and human (*C. trachomatis*) species is the ability to ascend to the upper genital tract using a mouse infection model: *C. trachomatis* is unable to ascend, whereas *C. muridarum* readily ascends leading to pathology. To investigate possible genetic factors associated with these phenotypes, we employed the ability of *Chlamydia* to undergo lateral genetic exchange between species. Genetic hybrids of *C. muridarum* and *C. trachomatis* were isolated and evaluated for their ability to ascend. Genomes of hybrids were then compared to parental strains to identify regions of genetic variance. Among the 35 isolated hybrids, 32 showed recombination within the same region, flanking but not incorporating the cytotoxins. Three isolates recombined two cytotoxins. Upon investigations *in vivo*, it was found these two cytotoxins were insufficient for ascension. Further investigations utilizing genetic hybrids may identify correlates of host- and disease-specificity and elucidate critical steps in chlamydial pathogenesis.

Chapter II.

Introduction

Genetic recombination is a central node for increasing diversity and promoting survival in all living things. Among eukaryotes, naturally occurring recombination events during meiosis and mitosis occur due to overlapping of sister chromatids during metaphase alignment (Marston and Amon 2004). Within bacteria, genetic recombination typically stems from horizontal gene transfer between microbes in which foreign, donor DNA become integrated into a recipient cell. This integration results in two different avenues of recombination: plasmid or chromosome transformation (Kidane, Ayora et al. 2012). In both, regions of sequence homology between processed donor ssDNA and recipient DNA allows for transient molecular interactions, the result of which leads to incorporation of the donor DNA. With the current advances in whole genome sequencing, it has been found that extensive homologous recombination occurs between widespread varieties of species and may serve as an essential contributor to the origination and virulence of many pathogens (Spratt, Hanage et al. 2001, Lawrence and Retchless 2009, Kong, Ma et al. 2013).

Less than a decade ago, it was discovered that *Chlamydia* is also capable of homologous recombination. *Chlamydia* is the leading bacterial sexually transmitted infection worldwide and the leading cause of non-heritable blindness worldwide. At present, mechanisms behind the complicated developmental cycle and pathogenesis, including the ability to ascend to the upper genital tract—the primary site for causing disease, remain poorly understood. Similarly, it has been observed that a fraction of individuals previously treated for *C. trachomatis* infections remain positive for infection at follow-up exams—potentially due to the organism's ability to

evade host immune responses and the presence of latent infections (Demars, Weinfurter et al. 2007). This evasion may, in part, be due to the organism's capability to undergo homologous recombination. Veritably, clinical isolates from urogenital tracts showed intergenic recombination among outer membrane protein-encoding genes *ompA* and *pmpC*; two proteins vital for immune recognition and clearance of the organism (Demars, Weinfurter et al. 2007). It is well known that chlamydial infections begin with an elementary body (EB) becoming endocytosed into a host cell and rapidly hijacking and altering the endocytic vacuole (inclusion) for its own use, such as evasion of lysosomal fusion (Fields and Hackstadt 2002). At high levels of infection *in vitro*, it has been observed that multiple EBs may be endocytosed, resulting in many inclusions present within one host cell (Bernkopf, Mashiah et al. 1962, Blyth and Taverne 1972, Ridderhof and Barnes 1989). Remarkably, it has been found that these numerous inclusions are capable of fusing with one another, resulting in one large inclusion containing all the endocytosed EBs (Ridderhof and Barnes 1989), while concurrently preventing lysosomal fusion. This ability allows for interaction of multiple EBs, potentially from different strains or serovars, facilitating the observed instances of homologous recombination (Hayes, Yearsley et al. 1994).

In *Chlamydia*, intentional co-infections *in vitro* using strains containing distinct antibiotic resistances and subsequently placing these organisms under dual-antibiotic selection has resulted in the isolation of recombinant progeny containing both resistance markers. Customarily, this application has been applied to *C. trachomatis* for the generation of novel, selectable isolates for experimental use (Demars, Weinfurter et al. 2007), however, Jeffrey et al. showed homologous recombination may also be applied to identify genotype-phenotype correlations (Jeffrey, Suchland et al. 2013). These studies showed recombination among the polymorphic membrane

proteins (Pmps) from *C. trachomatis* clinical isolates is associated with rectal tissue tropism and resultant *in vitro* generated *C. trachomatis* recombinants altered the organism's ability to attach to host cells and generate secondary inclusions in serovars F, J and L2 (Jeffrey, Suchland et al. 2010, Jeffrey, Suchland et al. 2013).

The study reported here, however, is the first to evaluate the ability for interspecies homologous recombination to occur between *C. trachomatis* and *C. muridarum*. These two species contain ~98% sequence identity, yet show drastically dissimilar pathogenesis in the mouse model of infection (Thomson, Holden et al. 2008, Stephens, Myers et al. 2009). *C. muridarum* is capable of ascending to the upper genital tract where it causes pathology following vaginal infection, as is observed with infections in women. *C. trachomatis*, on the other hand, is readily cleared following vaginal infections in mice, rarely ascending to the upper genital tract (UGT). Notwithstanding high genomic similarity between *C. trachomatis* and *C. muridarum*, diversity in pathogenicity may be the result of a specific region with substantial sequence variation, known as the plasticity zone (loci between *dsbB* and *ycrF* genes; (Figure 2.1 (Rajaram, Giebel et al. 2015)). Positioned roughly 12 kb to the right of the PZ lies the DNA gyrase subunit A (*gyrA* in *C. trachomatis*; *gyrA-2* in *C. muridarum*), in which *C. muridarum* is naturally ofloxacin resistant. This naturally occurring resistance is absent from *C. trachomatis*, however, spontaneous mutations have been detected *in vitro* leading to similar resistance (Suchland, Bourillon et al. 2005). Within the PZ itself, major differences include the presence of three large putative cytotoxins (~10 kb each) and a *guaBA* operon in the *C. muridarum* genome, while *C. trachomatis* lacks the cytotoxins and encodes a tryptophan synthesis operon in place of the *guaBA* (Rajaram, Giebel et al. 2015). Studies focused on this plasticity zone have revealed mutations within individual cytotoxins as well as the *guaBA* operon resulted in growth defects of

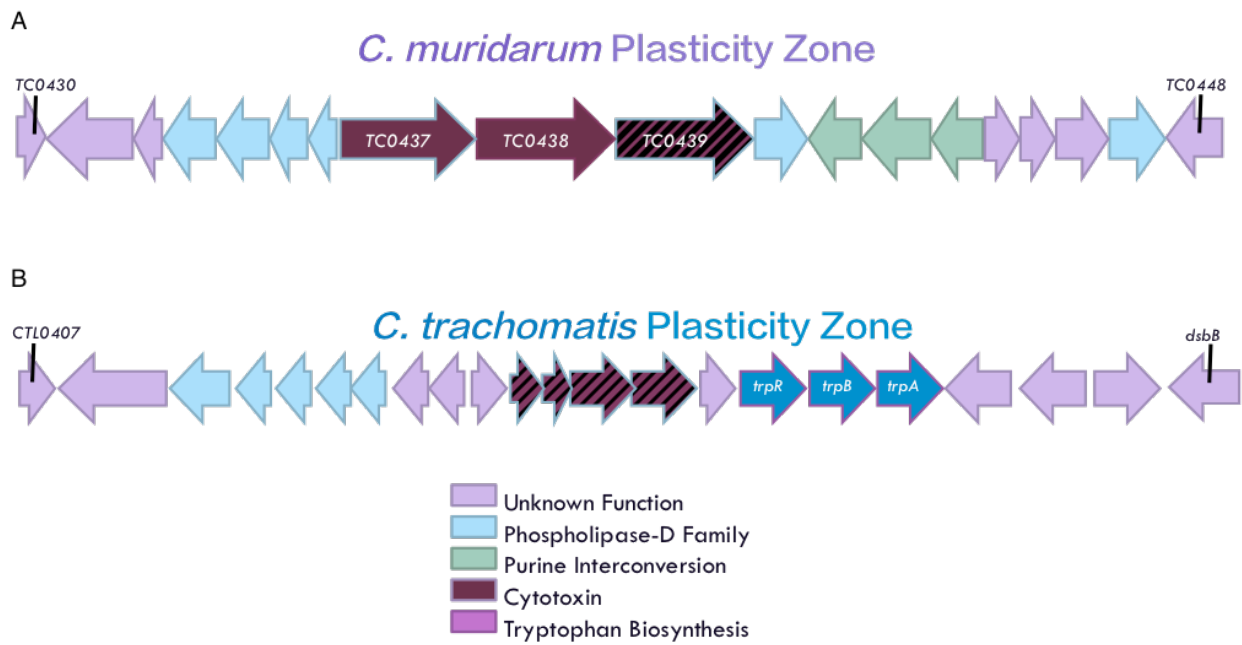


Figure 2.1 Schematic of *C. muridarum* and *C. trachomatis* Plasticity Zones (PZ). Map showing the genetic difference between plasticity zones. A) *C. muridarum* plasticity zone between *TC0430* and *TC0448*. The three large cytotoxins are highlighted in dark red. B) Plasticity zone of *C. trachomatis* between *CTL0407* and *dsbB*. Fragments of cytotoxin *TC0439* are shown in striped dark red. The tryptophan synthesis operon is shown in dark blue.

C. muridarum and single mutations in two of these cytotoxins (*tc0437* and *tc0439*, respectively) resulted in reduced toxicity *in vitro*, with decreased bacterial burden at early time-points of mammalian infection (Rajaram, Giebel et al. 2015). Given these initial observations, it is hypothesized that this region, particularly the cytotoxins within, are responsible for the substantial differences in mammalian pathogenesis. Unfortunately, the generation of double- and triple-mutants amongst the cytotoxins has proven to be a challenge. Similarly, the cloning and purification of recombinant cytotoxins using *E. coli* has been unsuccessful (Newman 2015).

In order to overcome these obstacles, homologous recombination was exploited. The generation of progeny consisting of a primarily *C. trachomatis* background, yet containing regions of the *C. muridarum* plasticity zone (i.e. one or more cytotoxins), provides an opportunity for discovery of a novel gain-of-function: *C. trachomatis* isolates capable of ascending to the UGT using a mouse vaginal model of infection. Relatedly, the introduction of *C. trachomatis* plasticity zone genes may result in loss of function recombinants: *C. muridarum* isolates incapable of ascension. Collectively, this research not only reports the first observations of recombination occurring between *C. trachomatis* and *C. muridarum*, but it also allows for identification of genetic regions interchangeable between them, narrowing the gap in understanding species-specific pathogenesis and host-tropism of *Chlamydia*.

Chapter II.

Methods and Materials

Chlamydial strains and selection for recombinants. All experiments with tetracycline-resistant *Chlamydia* described in this work were reviewed and approved by the National Institutes of Health Recombinant DNA Advisory Committee (University of Washington). Tetracycline and rifampicin dual-resistant *Chlamydia trachomatis* L2/434/Bu and ofloxacin resistant *C. muridarum* (Nigg) were generated as previously described (Figure 2.2 (Suchland, Sandoz et al. 2009)) and kindly provided by Robert J. Suchland at the University of Washington. Briefly, shell vials containing McCoy cells (ATCC CRL-1969) were infected with *C. trachomatis* and allowed to grow for ~8 hrs at which point *C. muridarum* was added at a combined MOI of 1. Cultures were allowed to infect 24-48 hours. Upon detection of growth, cells from individual shell vials were detached using -80°C/37°C freeze-thaw and split into 96 individual shell vials. Recombinants were selected using triple antibiotic selection with tetracycline, rifampicin, and ofloxacin and allowed to propagate until visible inclusions were detected. Vials containing detectable inclusions were then diluted 1:10 and split into 96-well plates and allowed to continue propagation under antibiotic selection. Per plate, roughly 1-5 wells showed chlamydial growth (Figure 2.3). These wells were then isolated and cloned via limiting dilution. *C. trachomatis* L2-specific monoclonal antibodies for IncA and OmpA were used to identify parental background flanking the plasticity zone.

PCR determination of recombination regions. In order to identify genetic regions of recombination near the plasticity zone, PCR was performed using either *C. trachomatis* or *C.*

muridarum species-specific primers (Table 2.1). DNA was isolated from samples by centrifuging aliquots, removing supernatant and boiling pellets in water at 95°C for 10mins. Standard Taq polymerase was used with the following PCR conditions: 95°C for 1 minutes; 35 cycles of 95°C for 30 seconds, 54°C for 30 seconds, 72°C for 1 minute; 72°C for 5minues, 4°C hold. PCR product was analyzed on 1.5% agarose gels ran at 90 volts. Amplicons were sent for sequencing using ACGT, Inc. (Germantown, MD).

Whole Genome Sequencing of Recombinants. In order to identify precise regions of genetic overlap, whole genome sequencing and contig assembly was kindly performed by Brian Brunelle (National Animal Disease Center, ARS, USDA, Ames, Iowa). Contigs were sent as FASTA files and BLASTed to identify the precise region of sequence overlap from each parent, *C. trachomatis* or *C. muridarum*. Table of genes lost, gained and replaced was generated for sample 3220 using the BLOSUM62 matrix with an E-value <1e-6 to identify orthologous genes (Pearson 2013).

Immunofluorescence microscopy. In order to determine infective titers for *in vivo* studies, IFM was performed as previously described (Osaka, Hills et al. 2012). Using 8-well ibiTreat μ -Slides (Ibidi, Martinsried, Germany), L929 cells were grown to confluency and infected with respective recombinant progeny. Cells were fixed with 100% methanol ~24 hpi, and washed three times with PBS. Slides were stained using either the MicroTrack *C. trachomatis* culture confirmation test (Syva Co., Palo Alto, CA) or Virostat *Chlamydia* MONOTOPE Fluorescein Conjugated IgG1 LPS, diluted in PBS at room temperature in the dark or overnight at 4°C overnight. 20 μ l of 1 μ M 4', 6-diamidino-2-phenylindole (DAPI) diluted in PBS was then added to wells and

Table 2.1: Primers used to determine recombination regions

<i>C. trachomatis</i> x <i>C. muridarum</i> PCR (5'-3')		
<u>Gene</u>	<u>Primer Sequence and Designation</u>	<u>PCR Product Size (bp)</u>
<i>C. trachomatis trpA</i>	GCTATCTAACCGCTGGTGATG TCTTCCAGCACCTTTATCACAC	347
<i>C. trachomatis dsbG</i>	GGATTAATTTCTCTTCCGGAATGCGTAGG GGTGCATAAGGATCCCCTATCGTAGG	362
<i>C. trachomatis gyrA/</i> <i>C. muridarum gyrA-2</i>	TCGATGGATCCAAAATTCCTTGACC CCTCTTCAAGATTTTTAGGGACGATG	318
<i>C. muridarum TC0437</i>	TTTGATCGGAGGGCATTTAG AAGTCCCTCGAAGCAACTCA	643
<i>C. muridarum TC0438</i>	CTGACGAGAGCACTTGGTGT TTCCCTTTCTTGTCCCGCTC	618
<i>C. muridarum TC0439</i>	GTCAGCGCTTTAGGATTGCG GGTCCTCAGCTCCAAAACGA	612
<i>C. muridarum TC0440</i>	CTTACCCATCCCGTCATTCTT CTCTTCTTCTCGAGGACGTTTC	405
<i>C. muridarum TC0495</i>	CTAAACCAGCATACCCCAAACATTCCG CCTCCATTCTTAATGTAGATTCTACATGCCCCAT	591

allowed to stain RT in the dark. Stain was then removed, and the cells washed with PBS. A final overlay of 0.1M tris-glycerol was added and cells were imaged using an Olympus IX81 inverted epifluorescence microscope. IFU/ μ L was calculated for each recombinant and used as the pre-infection titers.

In vivo mouse infections. Female C57BL/6 mice 6-8 weeks old were purchased from Jackson Laboratories and housed in accordance with the University of Kansas Institutional Care and Use Committee requirements. 2.5mg Medroxyprogesteronacetat (Depo-Provera, Pfizer, NY) was used to treat mice subcutaneously upon arrival (day -7). Unthawed aliquots of either WT *C. trachomatis*, WT *C. muridarum* or recombinant clones were diluted to a final dose of 1×10^4 IFU in sucrose-phosphate glutamic-acid (SPG) buffer and kept on ice until use. Using nasopharyngeal swabs (Puritan, Guilford, Maine), vaginal vaults were swabbed and the infective dose was administered using sterile tips in conjunction with a P10 pipette. Tips were inserted until reaching the cervix of the animals, slightly withdrawn and inoculated. Animals were held briefly with the tip still inserted to ensure no dose was secreted or pulled out upon removal of the pipette tip. L929 monolayers were infected with serial dilutions of each dose and stained 24 hpi as described above for calculations of post-inoculation titers. Data are shown as scatter dot plots with mean and standard deviation for 3 mice. Statistics were calculated using GraphPad Prism version 7.0b (La Jolla, CA).

ddPCR assessment of *Chlamydia* infection *in vivo*. Seven days post-infection, mice were humanely euthanized and the genital tracts were collected in SPG. Using a rotor/stator homogenizer (Biospec, Bartlesville, OK), organs were homogenized and 100uL aliquots were

frozen at -20°C until use. Remaining stocks of homogenized tissues were stored at -80°C. Using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), DNA was isolated from the 100µL aliquots and used for droplet digital PCR. Table 2.1 lists sequences for primers and probes for *Chlamydia rpoB* and mouse *rpp3*. ddPCR Supermix for Probes was used to set up PCR reactions and Oil-for-Probes droplet emulsions were generated using the droplet generator cassette (Bio-rad, California). The PCR conditions are as follows: 95°C for 10 minutes, 40 cycles of 94°C for 30 seconds and 60°C for 1 minute, and 98°C for 10 mins followed by cooling to 4°C. The QX200 droplet reader (Bio-Rad, California) was used to calculate fluorescent reads. Data was analyzed using the QuantaSoft Software (Bio-rad, California) and reported as a ratio of *Chlamydia* DNA to host DNA (*rpoB/rpp30*, copies/µL). Box and whisker scatter plots were generated in GraphPad Prism 7 and recoverable organisms were compared using unpaired, multiple t-tests with no correction for multiple comparisons.

Chapter II.

Results

Generation of *C. muridarum* and *C. trachomatis* recombinant progeny.

C. trachomatis and *C. muridarum* exhibit high sequence similarity (~98%), with majority of genetic variation occurring within the plasticity zone (PZ, Figure 2.1). It was thus proposed that these large differences were responsible for the radical dissimilarity observed during mammalian infections using a mouse model (Stephens, Myers et al. 2009). Given that this PZ encompasses 20-50kb due to the presence or absence of cytotoxins, the prospect of directed genetic disruptions remains unmanageable and, previous communications have demonstrated introducing *C. muridarum* PZ components into *C. trachomatis* are unsuccessful, particularly the introduction of the three large cytotoxins from *C. muridarum* (K. Fields, personal communication, CBRS, 2017). In order to investigate how this region may be playing a role in the differentiation in pathogenesis, the natural ability of *Chlamydia* to undergo lateral gene transfer was exploited. Both species were used to co-infect monolayers at high MOIs, likely resulting in multiple inclusions per cell. The fusion of these inclusions allows for direct interaction of *C. trachomatis* and *C. muridarum* and the exchange of genetic material, resulting in hybrid progeny containing loci from each species.

Recombinant progeny were isolated through triple antibiotic selection using tetracycline, rifampicin and ofloxacin (Figure 2.2). As can be seen from the diagram, it is postulated that ofloxacin resistance came from the naturally resistant *C. muridarum gyrA* mutation, whereas tetracycline and rifampicin resistance (due to mutations in *rpoB*) originated from *C. trachomatis*. These respective markers were selected not only because of their availability but also their

location around the genome. Through this selection, progeny containing a primarily *C. trachomatis* backbone would be enriched for, with recombination occurring near the plasticity zone to include components from *C. muridarum*. Given that the distance between the tetracycline marker and the rifampicin marker is rather large, it cannot be ruled out that multiple regions of recombination may have occurred between them. In order to evaluate this, immunofluorescence microscopy was performed. *C. trachomatis*-specific antibodies reactive to the major outer membrane protein, OmpA, were used due to the gene's positioning between the *tet* and *rif* resistance markers (Figure 2.2). *C. trachomatis*-specific antibodies reactive to IncA were also used due to the gene's proximity to the plasticity zone; IncA lies to the right of the Tet Island, just left of both the plasticity zone and *gyrA*, providing an "endpoint" for recombination in this region—i.e. components from *C. muridarum* integrated between IncA and the rif-resistance marker. Through these selective approaches, isolated progeny should contain a primarily *C. trachomatis* backbone (counter-clockwise: IncA, tet-resistance, OmpA and rif-resistance), with regions of *C. muridarum* near the plasticity zone, between rif-resistance and IncA, including *gyrA*, Figure 2.2).

Recombination occurs at a very low frequency.

Previous *in vitro* experiments in which two different *Chlamydia* species, *C. suis* and *C. trachomatis*, were permitted to recombine resulted in low recombination frequencies. Therefore it was expected that *C. muridarum* and *C. trachomatis* experiments would show higher frequencies of recombination due to more genetic similarity, but still low overall rates (Suchland, Sandoz et al. 2009). Due to the faster growth rates of *C. muridarum*, cultures were initially infected with *C. trachomatis* and grown 8 hrs prior to the addition of *C. muridarum*. Each vial

■ *C. trachomatis* LGV ■ *C. muridarum* Nigg

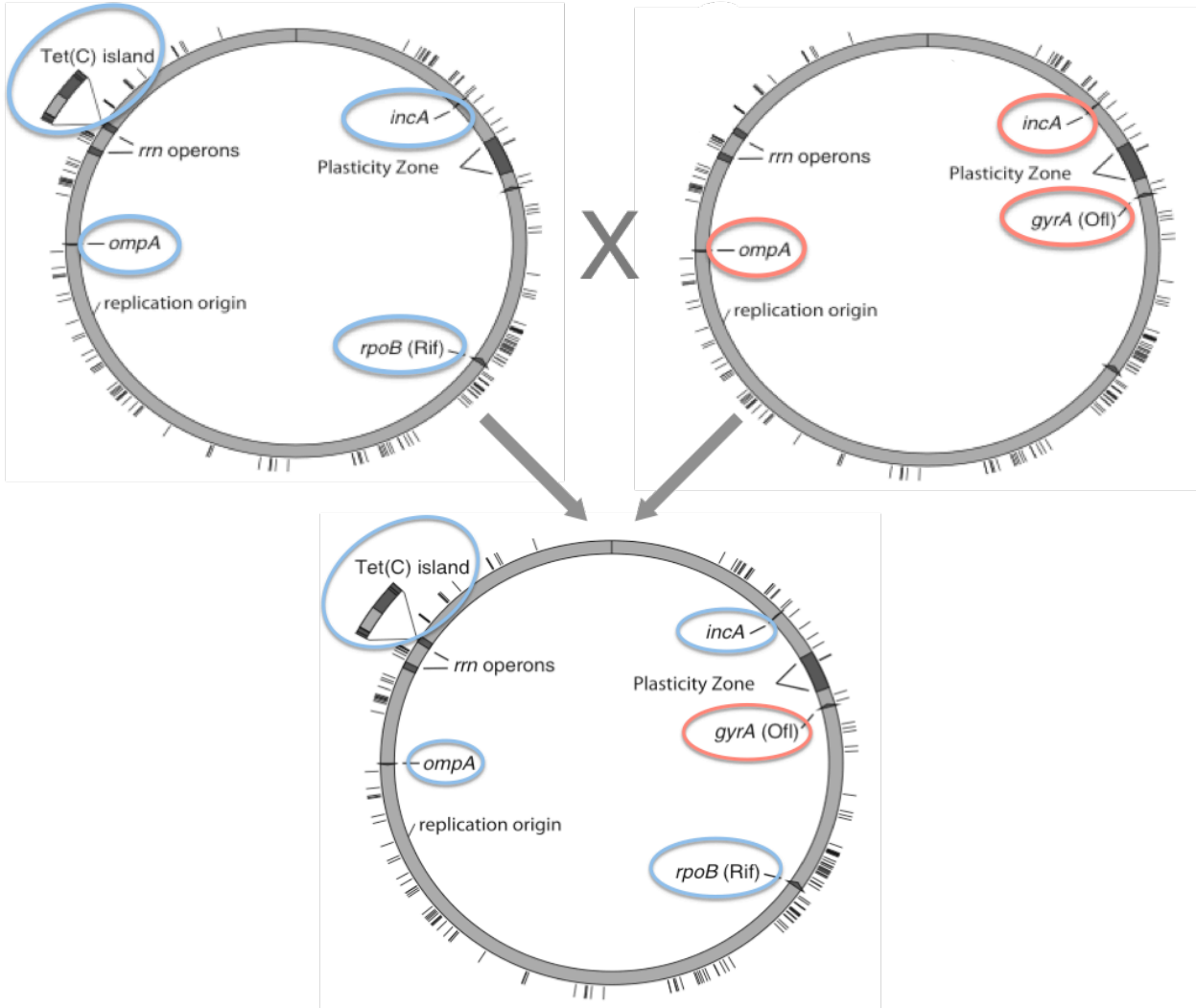
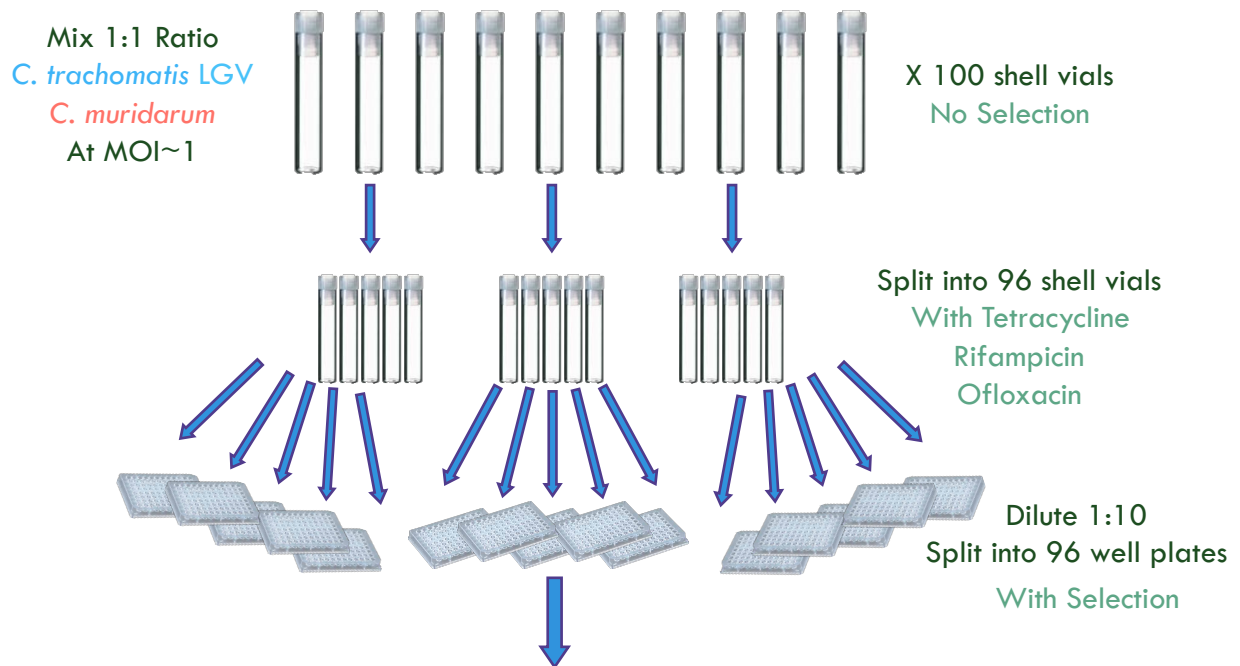


Figure 2.2 Antibiotic resistances of parental and recombinant progeny. The generation of recombinant progeny was done using co-cultures of tetracycline and rifampicin resistant *C. trachomatis* LGV (blue) and ofloxacin resistant *C. muridarum* Nigg (red). After incubation without selection for 48 hours, cultures were treated with all three antibiotics and monitored for progeny growth. Survival indicated isolates contained all three antibiotic markers. The location of the *C. muridarum* ofloxacin resistant marker shows recombination occurred near or within the plasticity zone (PLZ). Given the low frequency of recombination events, it is expected the distance between the tetracycline and rifampicin resistance markers is *C. trachomatis* LGV origin, resulting in a predominantly LGV genome.

contained about 8.6×10^6 IFU of *C. trachomatis* and *C. muridarum* combined (Figure 2.3) which was then treated with antibiotics and diluted into 96-well plates. In spite the high initial titers, each experiment yielded an average of ~1 well containing recombinant, triple-antibiotic resistant progeny (range 0-7).

Spontaneous mutation in *gyrA* results in ofloxacin resistant *C. trachomatis*.

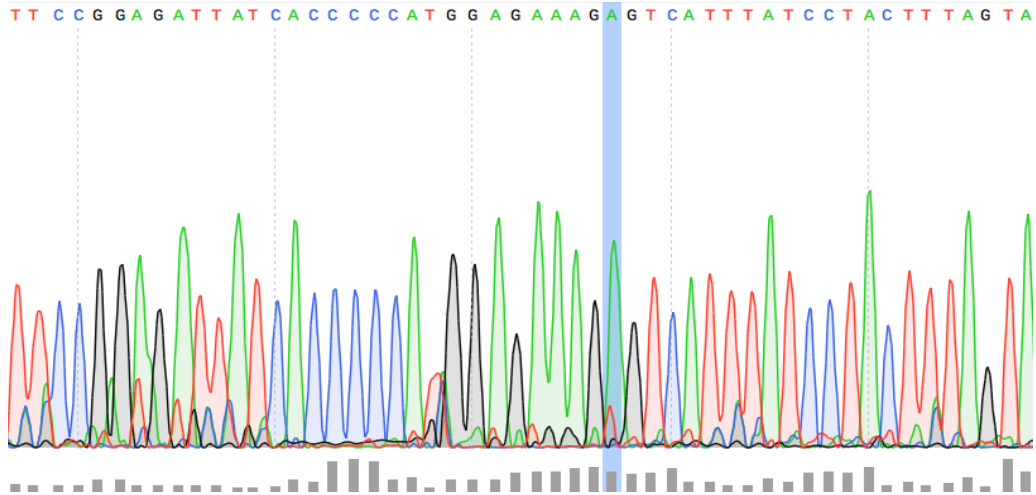
As a result of various recombination experiments, 65 clones have been harvested and initially characterized via PCR to determine whether ofloxacin resistance is due to the presence of *C. muridarum gyrA* acquired through recombination or a spontaneous mutation in *C. trachomatis gyrA*. Considering that all harvested isolates are resistant to ofloxacin, it is expected *gyrA* from each would sequence to that of *C. muridarum*. In order to streamline experiments, primers were designed at conserved regions within *gyrA* to bind both *C. muridarum* and *C. trachomatis*. Internal sequence, however, differed enough such that sequencing of amplicons allowed for discrimination between each species. Unexpectedly, numerous samples sequenced to *C. trachomatis gyrA*, leading to the investigation of possible spontaneous mutations resulting in ofloxacin resistance. Indeed, the presence of a T→A mutation and resulting conversion of serine to arginine at position 83 was identified (Figure 2.4). It has been previously shown that similar mutations confer resistance to fluoroquinolones in *E. coli* and *Staphylococcus aureus*, albeit substituting leucine rather than arginine (Sreedharan, Oram et al. 1990, Bagel, Hüllen et al. 1999, Barnard and Maxwell 2001); therefore this comparable mutation is likely having the same effect. In total, it was discovered that 29 clones sequence *gyrA* from *C. trachomatis*—nearly 45% of the library.



From ONE 96-well plate, typically 1-5 wells show *Chlamydia* growth

Figure 2.3 Schematic showing recombination protocol. For the generation of recombinant progeny, the following scheme was utilized. Equal ratios of antibiotic resistant *C. trachomatis* LGV and *C. muridarum* Nigg were used to infect monolayers in shell vials at an MOI of 1 without selection. After 24-48 hrs, individual shell vials were split into 96 new vials containing host cells, and treated with triple antibiotic selection. Upon observation of recombinant progeny growth, individual vials were diluted and split into 96-well plates and monitored for growth. As indicated, one plate typically generates 1-5 wells of progeny, highlighting the extremely low frequency of recombination. Isolates from the 96-well plates were clonally diluted and used for subsequent analyses.

A



B

MLNKEEII VPKNLEEMKESYLRYSMSV IISRALPDARDGLKPSQRRILYAMQNLNTPGVKHKRCAKICGDTSGDYHPHGE**R**
VIYPTLVRMAQDWAMRYPLVDGQGNFGSIDGPAAMRYTEARLTHSAIFLLEDLDKDTVDMVPNYDETKYEPVVFPSKFPNL
LCNGSSGI AVGMATNIPPHNLGELIEATLLVLANSQTSIEDILEVMGPDFPTGGICGTEGIRSTYYTGRGKLRARMHVE
ENSDKQRENIILTEMPYVNVKSRLIEQIAELINEKTLTGISDVRESDKDGIRVVLELKKGESSEVVINRLYKFTDVQVTFGA
NMLALDKNLPRTMNIHRMISAWIRHRMDVIRRRTRYELNKAERAHILEGFLKALSCMDEVVKTIRESSNKEHAKQQLVELFS
FSEAQALAILRLRYQLTGLEADKVQKEYSELLEKITYYRKVLAEELVKDIIREELQELHKVHKTPRRTKIEMDTGDVVDIE
DIIISDESVIITISGDDYVKRMPVKVFREQRGGQGVTFDMKKGDFLKAVYSASTKDYLIIFTNFGQCYWLKVWQLPEGERR
AKGKPIINFLEGIRPGEQVAAVLNVKRFEQGEYLFLLATKKGVVKKVSLDAFGSPRKGIRALEIDDGDELIAARHIANDEEKV
MLFTRLGMAVRFPHDKVRPMGRAARGVRSLSKNEQDFVVSQVVTEDQSVLVVCDNFGKRSVLCDFRETNRGVSIVRSIVI
NQRNGDVLGAI SVTDCDSILLMSAQQAIRINMQDVRVMGRATQGVRLVNLREGDTLVAMEKLSINTESVETEENLAASVQSG
QDTIEE

Figure 2.4 Identification of *gyrA* spontaneous mutation. Genome sequencing of *gyrA* PCR product revealed sequences from several clones aligning to *C. trachomatis gyrA*. A) Analysis of sequence demonstrated a point mutation at nucleotide 202 resulting in T→A conversion (blue) changing codon AGT → AGA. Quality values for each base are graphically represented below the chromatogram (gray). B) Analysis of this change in reference to the protein sequence resulted in a Serine to Arginine conversion at amino acid 83 (highlighted in yellow).

Identification of recombination regions.

While the subset of clones containing the spontaneous mutation may still be chimeric, efforts to characterize the library were shifted to focus on those samples definitively recombinant, as determined by the presence of *C. muridarum gyrA-2* sequence. In order to identify if any part of the *C. trachomatis* plasticity zone was present, genes specific to the tryptophan synthesis operon were assessed. Primers for *trpA* were designed and PCR on the remaining 35 isolates was performed (Table 2.2). For nearly every sample a band was observed, proposing that genome regions from *incA* to *trpA* are of *C. trachomatis* origin. Primers further downstream of *trpA* were designed to amplify *dsbG*, however, no bands amplified for any samples, signifying a cross over region between *trpA* and *dsbG* in which the genome becomes *C. muridarum*.

As the central hypothesis emphasizes the role of cytotoxins in pathogenic diversity between *C. muridarum* and *C. trachomatis*, PCRs determining whether chimeric isolates contain the cytotoxins were assayed. Primers amplifying fragments of *TC0437*, *TC0438*, and *TC0439* were used on each of the *gyrA-2* positive clones. It was found that among the 35 samples, only 3 amplified bands for *TC0438* and *TC0439* (Figure 2.5). All three samples showed bands at similar sizes to the parental *C. muridarum* control and as expected, no bands are present in the *C. trachomatis* parental samples. None of the isolates amplified *TC0437*. These three samples were then propagated and used for analyses *in vivo*.

The remaining 32 samples were assessed for recombination beyond the absent cytotoxins. Interestingly, of the 27 samples tested, 23 showed bands amplifying *TC0440*, suggesting the genome spanning from *TC0440* to *gyrA-2* (*TC0461*) originated from *C. muridarum*. As mentioned above, the majority of samples did not amplify for *TC0495*, suggesting a

Table 2.2 Parental Gene/Species Identified in Recombinant Isolates

Clone #	<u><i>trpA</i></u> <u>(<i>C. trachomatis</i>)</u>	<u>Cytotoxins</u> <u>(<i>C. muridarum</i>)</u>	<u><i>TC0440</i></u> <u>(<i>C. muridarum</i>)</u>
61	Yes	No	Yes
88	Yes	No	Yes
89	Yes	No	Yes
105	Yes	No	Yes
107	Yes	No	Yes
112	Yes	No	Yes
126	Yes	No	Yes
136	Yes	No	Yes
149	N.D.	No	N.D.
155	Yes	No	No
408	Yes	No	Yes
501	Yes	No	Yes
522	Yes	No	Yes
672	Yes	No	Yes
871	Yes	No	Yes
940	Yes	No	Yes
986	Yes	No	N.D.
1299	N.D.	No	N.D.
1521	Yes	No	No
1790	Yes	No	No
1970	N.D.	No	N.D.
2043	Yes	No	Yes
2562	N.D.	No	N.D.
2697	N.D.	No	N.D.
2845	N.D.	No	N.D.
3106	Yes	No	Yes
3220	Yes	No	Yes
3401	No	No	N.D.
3710	Yes	No	Yes
4375	Yes	<i>TC0438, TC0439</i>	Yes
4406	Yes	<i>TC0438, TC0439</i>	Yes
4537	Yes	<i>TC0438, TC0439</i>	Yes
4665	Yes	No	Yes
5053	Yes	No	No
5254	Yes	No	Yes

N.D. Not Determined

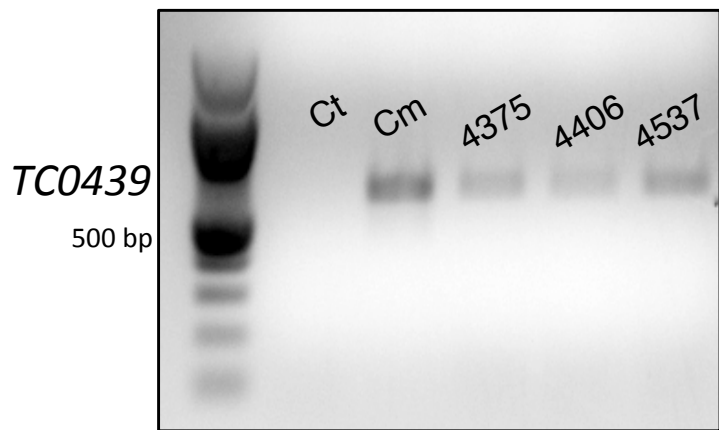
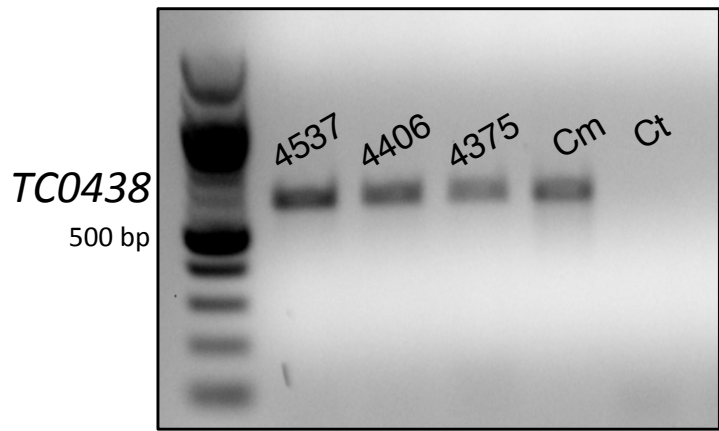
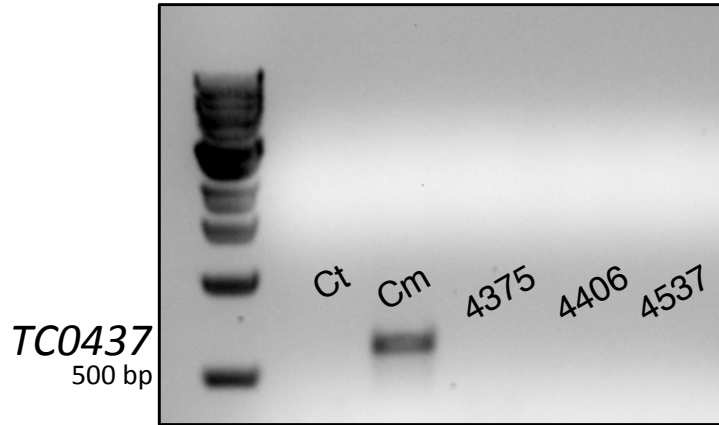


Figure 2.5 Isolates 4357, 4406, and 4537 contain two cytotoxins from *C. muridarum*.

PCRs using primers specific to the three cytotoxins from *C. muridarum*, TC0437, 8 and 9 were used. Ct=Wild type *C. trachomatis* parent, Cm=Wild type *C. muridarum* parent. All three cytotoxins amplify ~600bp (table 2.1). *C. trachomatis* does not amplify any cytotoxins while bands are present for all three in the *C. muridarum* samples. Corresponding bands at the same size are observed for all three samples when amplified using TC0438 and 9 primers, signifying these samples contain two of the three cytotoxins.

recombination occurred between *TC0461* and *TC0495*. This region encompasses over 30 genes and greater than 50kb of sequence. It was determined at this point that genome sequencing should be implemented to determine precise regions of recombination. Three samples, 3220, 3106, and 2043 were randomly selected, grown to high titer, and DNA isolated for sequencing. Samples were sent to Dr. Brian Brunelle at the USDA who kindly provided the whole genome sequencing. Contigs were generated and returned for analysis of genomes. Upon BLASTing sequences, it was found that for all three, recombination occurred within an intergenic span between *trpA* and *TC0440* (Figure 2.6). The second recombination region, though, differed greatly between them. The first and second samples, 3220 and 3106, had recombination points within coding regions of genes from each species. Sample 3220 had recombination in *CTL0444*: a *C. trachomatis* inclusion membrane protein and *TC0464*: a hypothetical protein from *C. muridarum*. Table 2.3 shows the composition of genes between each recombinant region in isolate 3220. Whereas only two *C. trachomatis* genes were lost (*CTL0425/6*), six novel genes from *C. muridarum* were incorporated, including three genes with no discernable *C. trachomatis* orthologs. The majority of genes were annotated to functionally replace those from *C. trachomatis*, likely retaining their respective roles in growth and infectivity (Table 2.3).

Sample 3106 showed recombination within the DNA gyrase subunit B from both species, resulting in similar genes gained/lost as 3220, except the *C. muridarum* thymidylate kinase (*tmk*) and partial *TC0464* (Figure 2.6B) The third and final sample, 2043, had the largest portion of *C. muridarum* sequence with the second region of recombination occurring intergenically between *TC0470* and *oppA3*. Whereas this intergenic overlap and the combination of *gyrB2* from each species in sample 3106 likely did not disrupt any gene functions, the mosaic outcome between *CTL0444* and *TC0464* from sample 3220 may have drastic effects within the

cell. Future studies wherein more comprehensive analysis into whether this chimeric gene produces a functional protein and/or if possible domains are retained between fragments of *CTL0444* and *TC0464*, or whether this fusion results in severe downstream effects will be advantageous for the investigation of roles these genes may have.

Recombinant clones do not ascend to the upper genital tract during mammalian infection.

As mentioned above, *C. muridarum* strains are capable of ascending to the upper genital tracts (UGT) of mice after a low-dose vaginal infection. *C. trachomatis* strains are not capable of this, instead requiring much higher doses and resulting in more rapid clearance of infection. It was postulated that cytotoxins present only in *C. muridarum* are responsible for this difference—thus the existence of these genes within a *C. trachomatis* genetic background may result in hybrids capable of ascension. Through techniques directing recombination near or within the plasticity zones of each species, three *C. trachomatis* hybrids were obtained encoding cytotoxins *TC0438* and *TC0439*. *In vivo* experiments were done to determine whether these cytotoxins confer the ability to ascend to the UGT of mice after vaginal infection with low titers. Parental *C. muridarum* and *C. trachomatis* strains used for the generation of these hybrids were included as positive and negative controls for ascension, respectively. As these experiments were simple “yes vs. no” experiments in terms of ascension, only three animals were used per group. One hybrid clone showing recombination within the plasticity zone that did not include acquisition of the cytotoxins was also included (sample 2043). Whole genome sequencing data was obtained for this isolate; therefore altered phenotypes due to procurement of new genes via recombination, along with the precise region of homology can be identified (Figure 2.6C).

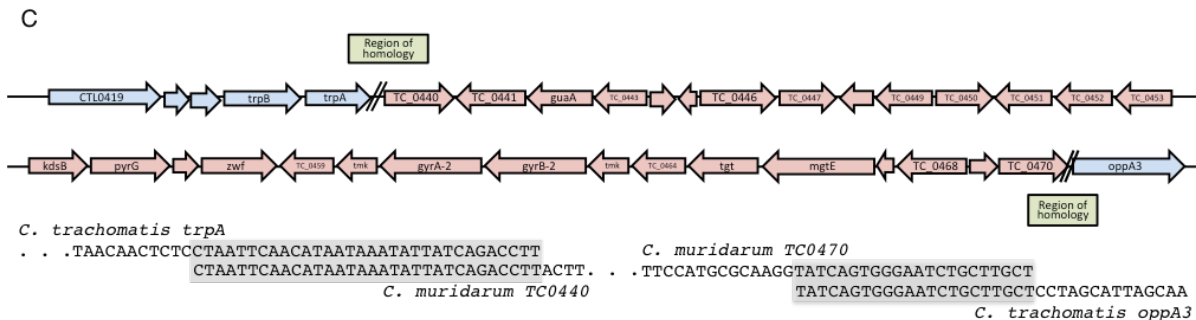
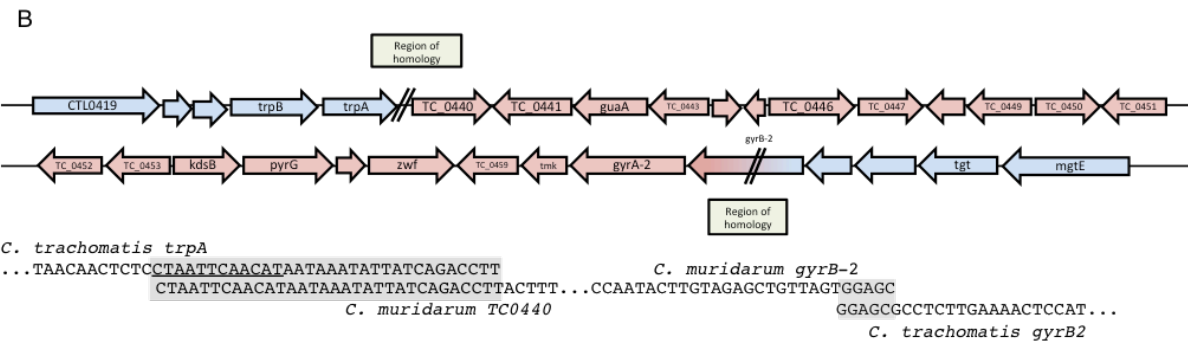
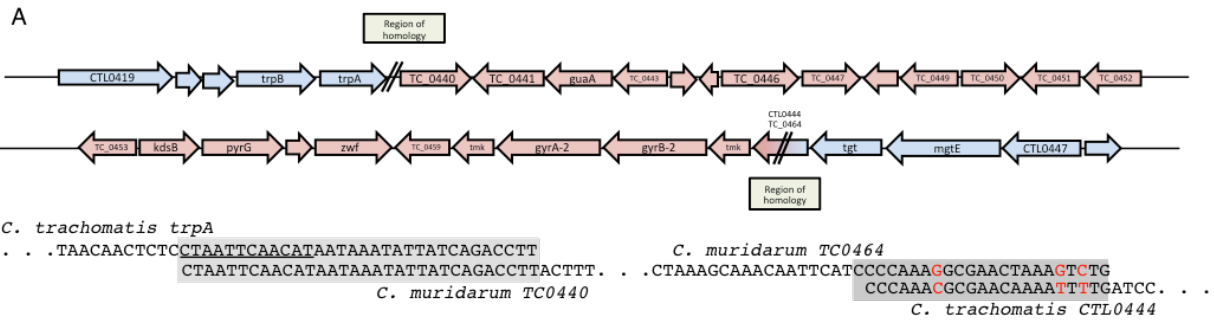


Figure 2.6 Schematic of recombination regions based on whole genome sequencing.

Whole genome sequencing was done on three hybrid samples. Based on BLAST analysis, regions of homology and likely crossover for recombination were identified (gray). All three samples showed the same crossover event at *trpA* and *TC0440*. A) Sample 3220 showed homologous sequence within *CTL0444* and *TC0464*. B) Sample 3106 showed homology within *gyrB2*, resulting in a chimeric gene. C) Sample 2043 demonstrated the largest span of *C. muridarum* sequence with recombination occurring at an intergenic region between *TC0470/0471* and *oppA3*. Blue=*C. trachomatis*; Red=*C. muridarum*.

Table 2.3. Summary of genome alterations following *C. muridarum* and *C. trachomatis* lateral gene transfer

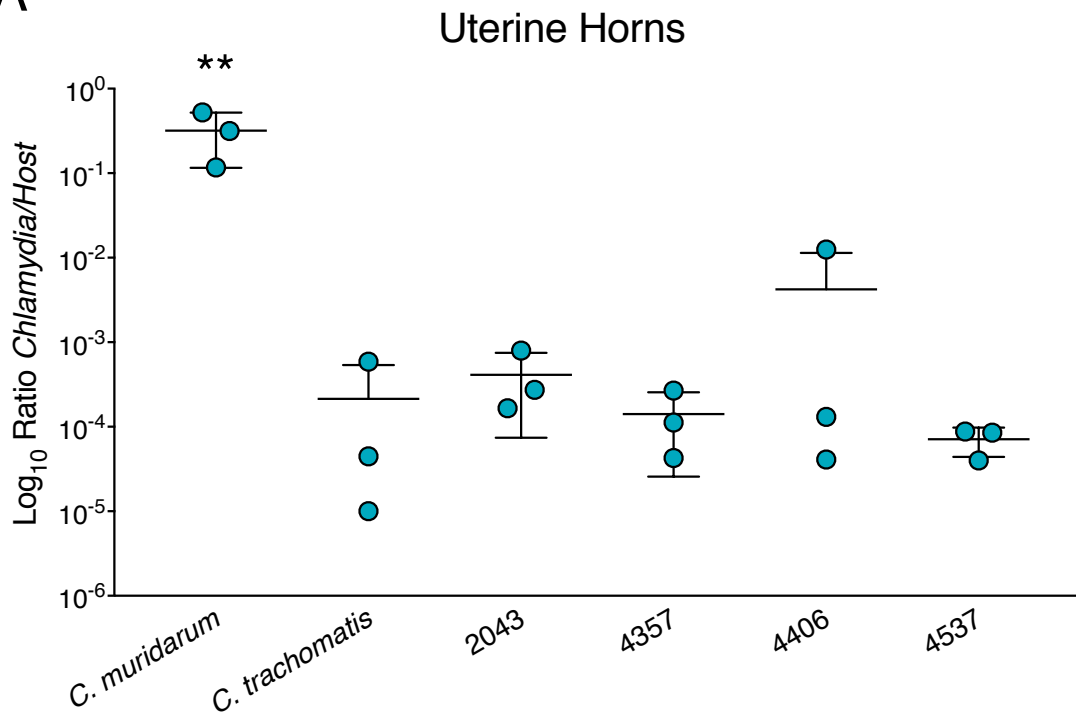
Unique <i>C. trachomatis</i> genes lost		Unique <i>C. muridarum</i> genes acquired	
<u>Gene</u>	<u>Function</u>	<u>Gene</u>	<u>Function</u>
<i>CTL0425</i>	Hypothetical, pseudogene	<i>TC0441</i>	Hypothetical
<i>CTL0426</i>	Hypothetical	<i>guaA</i>	GMP synthase
		<i>TC0443</i>	Inosine-5'-monophosphate dehydrogenase
		<i>TC0445</i>	Hypothetical
<i>C. muridarum</i> genes acquired potentially replacing <i>C. trachomatis</i> deleted ortholog			
<u>Gene</u>	<u>Function</u>	<u>Deleted ortholog in <i>C. trachomatis</i>^a</u>	
<i>TC0446</i>	Peptide ABC transporter	<i>CTL0427 (oppA2)</i>	
<i>TC0448</i>	dsbB	<i>CTL0428 (dsbB)</i>	
<i>TC0449</i>	dsbG	<i>CTL0429 (dsbG)</i>	
<i>TC0450</i>	Hypothetical	<i>CTL0430</i>	
<i>TC0451</i>	Hypothetical	<i>CTL0431</i>	
<i>TC0452</i>	ABC transporter	<i>CTL0432</i>	
<i>TC0453</i>	Hypothetical	<i>CTL0433</i>	
<i>ksdB</i>	3-deoxy-manno-octulosonate cytidyltransferase	<i>CTL0434 (kdsB)</i>	
<i>pyrG</i>	pyrG	<i>CTL0435 (pyrG)</i>	
<i>TC0456</i>	Holliday junction resolvase	<i>CTL0436</i>	
<i>zwf</i>	Glucose-6-phosphate 1-dehydrogenase	<i>CTL0437 (zwf)</i>	
<i>TC0458</i>	Glucosamine-6-phosphate isomerase	<i>CTL0438 (devB)</i>	
<i>TC0459</i>	DNA polymerase III, tau subunit	<i>CTL0439 (dnaX)</i>	
<i>tmk</i>	Thymidylate kinase	<i>CTL0440 (tmk)</i>	
<i>gyrA-2</i>	gyrA	<i>CTL0441 (gyrA)</i>	
<i>gyrB-2</i>	gyrB	<i>CTL0442 (gyrB)</i>	
<i>TC0463</i>	Hypothetical	<i>CTL0443</i>	
<i>TC0464</i>	Inclusions membrane protein	<i>CTL0444</i>	
<i>C. muridarum</i> genes acquired with existing orthologs in <i>C. trachomatis</i>			
<u>Gene</u>	<u>Function</u>	<u>Existing orthologs in <i>C. trachomatis</i>^a</u>	
<i>TC0440</i>	Phospholipase D family protein	<i>CTL0339, CTL0411, CTL0413, CTL0414a</i>	
<i>TC0444</i>	Hypothetical	<i>CTL0418, CTL0419</i>	
<i>TC0447</i>	Phospholipase D family protein	<i>CTL0339, CTL0411, CTL0413, CTL0414a</i>	

^aBLOSUM62 matrix E-value <1e⁻⁶

Cervix and uterine horns were harvested seven days post-infection and bacterial burden within each organ was assessed (Figure 2.7). All animals infected with parental *C. muridarum* had high levels of detectable bacteria in the uterine horns as expected, while *C. trachomatis*-infected animals had levels over four-log lower (Figure 2.7A). One animal infected with recombinant clone 4406 showed bacterial levels over 10^{-2} in the uterine horns, but the other two animals from that group showed levels below WT *C. trachomatis*-infected animals, bringing the group average down. Overall, the recombinant and *C. trachomatis*-infected animals showed significantly lower levels of bacteria in the uterine horns as compared to *C. muridarum* ($p < 0.01$).

Considering that organisms were deposited directly into the vagina (abutting the opening of the cervix), these tissues provide evidence for the ability to establish and maintain infection, even if bacteria are unable to ascend. As shown in Figure 2.7B, mean values for *C. muridarum* and isolate 4357 were the highest at roughly 1, while cervix values for animals infected with 4406 fall one-log below these. *C. trachomatis* and 4537-infected animals were detected at roughly two-log below *C. muridarum* at $\sim 10^{-2}$. A relative level of variation was observed among these groups spanning nearly 3-logs, which may be indicative of animals clearing infection. Unexpectedly, animals infected with recombinant isolate 2043 were the lowest and showed no real variation in detection. This clone does not encode any of the cytotoxins but has the largest region of *C. muridarum* sequence as determined by whole genome sequencing (Figure 2.6C). It could be that *C. muridarum* sequence replaced *C. trachomatis* genes imperative for establishing infection (genes between *trpA* and *oppA3*). To determine this, future studies utilizing timepoints to identify when bacterial clearance occurs should be implemented. Similarly, evaluations with larger sample sizes may provide more conclusive data with less inconsistency between animals.

A



B

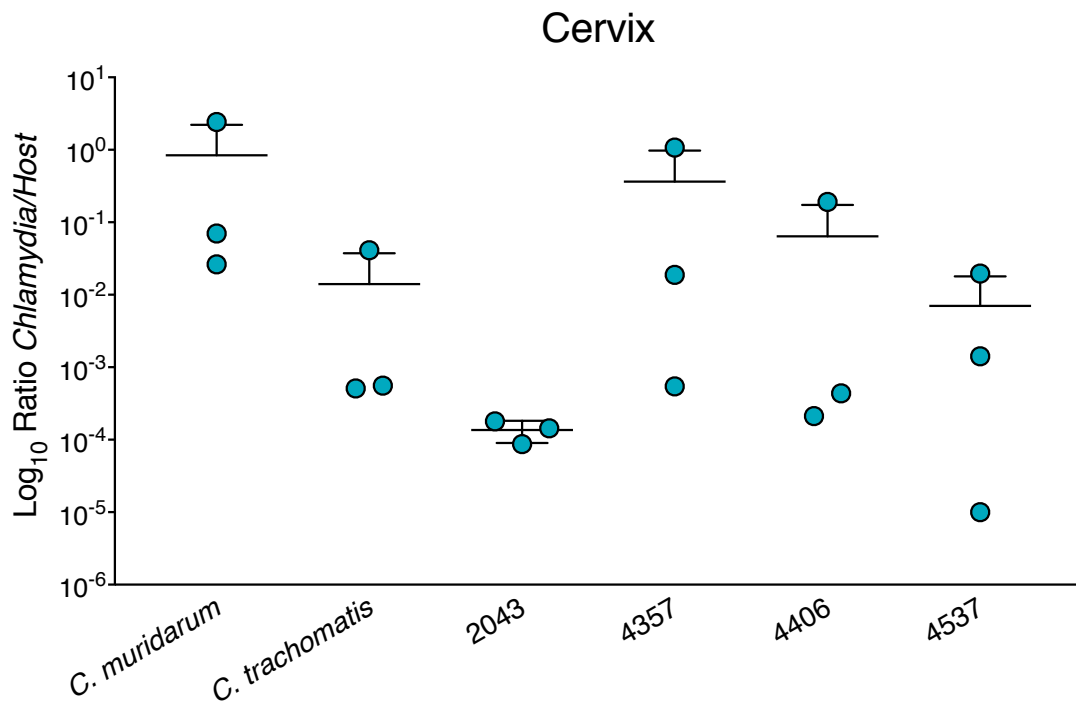


Figure 2.7 Vaginal infections with recombinant clones. Three recombinant clones PCR-amplified two cytotoxins from *C. muridarum*: *TC0438* and *TC0439*. To assess whether these confer the ability to ascend, vaginal infections with low doses (1×10^4 IFU) were used. Seven days post-infection, genital tracts were isolated and ddPCR was used to determine bacterial burdens. A) Presence of detectable *Chlamydia* in Uterine horns indicates the ability of organisms to ascend from the vaginal vault to the upper genital tract. B) Cervix samples verify infectivity and identify possible clearance of infection. Data are shown as individual scatter dot plots with log mean and S.D. for 3 mice. $**p < 0.01$ ANOVA with Tukey post-test.

Chapter II.

Discussion

Within nearly all bacterial species, homologous recombination has been shown to contribute to the vast genetic diversity and virulence of a number of pathogens (Lawrence and Retchless 2009) including *C. trachomatis*. Previous studies both *in vitro* and using clinical isolates have identified evidence of recombination occurring in outer membrane proteins (Gomes, Bruno et al. 2004), and the horizontal transfer of antibiotic resistance markers between species (Suchland, Sandoz et al. 2009). Within the past decade, the use of recombination as a means to associate genetic loci with certain phenotypes has increased dramatically, leading to the identification of specific genes contributing to chlamydial tissue tropisms (Jeffrey, Suchland et al. 2010). Although studies have predominantly focused on genetic exchange within serovars, the potential for transfer between species provides an enhanced capacity for phenotypic assessments. It has been shown that tetracycline resistance genes from *C. suis* are capable of recombining into the *C. trachomatis* genome; yet, it appears that few other genes are freely exchanged, potentially due to a lack of genetic compatibility (Suchland, Sandoz et al. 2009). Moreover, the investigations of whether recombination occurs between *C. trachomatis* and other species such as *C. muridarum*, *C. pneumoniae*, or *C. psittaci* have not previously been implemented.

This study marks the first report of intentional homologous recombination between *C. trachomatis* and *C. muridarum*. In spite of highly similar genetic content, these species show dramatically contrasting pathogenesis in a mouse model of infection. The plasticity zone (PZ), a locus with the greatest genetic diversity, is then inferred to be responsible for these disparities in pathogenesis—more specifically, three large putative cytotoxins present in *C. muridarum* that

are absent from *C. trachomatis* (Figure 2.1). To assess whether these genes, and/or genes within this locus are playing a role in infectivity, directed recombination using antibiotic resistance markers flanking the PZ was exploited. *C. muridarum* is naturally ofloxacin resistance in *gyrA-2*, the gene positioned left-flanking the PZ, while *C. trachomatis* strains used in this study are both tetracycline and rifampicin resistant. (Figure 2.2). Co-culturing both species and selecting with all three antibiotics allows for isolation of recombinant progeny likely comprising a predominantly *C. trachomatis* genome, possessing *C. muridarum* genes from regions near *gyrA-2*. *C. trachomatis* genetic composition was further enriched through the selection of progeny staining positively with *C. trachomatis* IncA and OmpA antibodies. Taken together, these features should result in hybrids containing *C. muridarum* genes spanning *incA* to *rpoB*, the gene responsible for rifampicin resistance in *C. trachomatis* (Figure 2.2). Perhaps not surprisingly, it was found that the generation of these recombinants was highly inefficient. Initial titers from co-cultures were $\sim 10^7$ IFU/vial at which point all three antibiotics were added (Figure 2.3) however splitting these cultures among 96-well plates resulted in a range of 0-7 wells containing triple-resistant isolates (Table 2.2). Further, it was determined that among those isolates selected for subsequent characterization, nearly half of the clones contain a spontaneous mutation in *gyrA* from *C. trachomatis*, rendering cultures resistant to ofloxacin. This antibiotic resistance marker is the only indicator of *C. muridarum* genes; therefore it is likely most, if not all, of these spontaneously resistant clones are not hybrids but rather wholly *C. trachomatis*.

Due to the high rate of spontaneous *gyrA* mutation, emphasis was placed on characterizing those mutants with *C. muridarum gyrA-2* sequence. The principal hypothesis highlights the *C. muridarum* cytotoxins, which are lacking in *C. trachomatis*, as the basis for differences in pathogenesis; so the presence of these genes was assessed. Shockingly, <10% of

the isolates tested amplified cytotoxin sequence. Moreover, all three of the cytotoxins are not present in any sample. Instead only two of the three, *TC0438* and *TC0439*, amplified. As shown in Figure 2.1, the *C. trachomatis* genome contains fragments purportedly from *TC0439*, annotated as pseudogenes. Consideration was taken in designing primers such that these fragments would not amplify. *In silico* PCR was used to verify primers will not amplify anything from *C. trachomatis*, confirming the presence of *C. muridarum*-derived *TC0439* (San Millán, Martínez-Ballesteros et al. 2013) .

These three isolates encoding *TC0438* and *TC0439* were then used to vaginally infect mice and assessed for the ability to ascend to the UGT. Only one animal infected with isolate 4406 showed detectable *Chlamydia* levels in the uterine horns approaching those observed with *C. muridarum*, however, other animals in this group did not show similar burdens (Figure 2.7). It cannot be disregarded that this highly infected animal may be the result of technical error during administration of the infective dose. Applying the dose too closely to the cervical opening may unintentionally promote deliverance into the UGT. The presence of a similar infection profile in the corresponding cervical data indicates that, indeed, this is likely due to inaccuracy during dosing. Nearly all groups show a gradual reduction in detectable organisms from the cervix. This may be indicative of infection clearance with all animals reaching peak infection levels between 10^{-2} and 1 at earlier timepoints. Overall, future studies using larger samples sizes as well as varying time of harvest will identify whether the observed data in 4406 is an artifact of administration or a true gain-of-function, and whether 2043 clears from mammals rapidly or if this was also a dosing issue.

Perhaps the most noteworthy outcome from analyzing recombinant clones was the observation that transfer of cytotoxins from *C. muridarum* into a *C. trachomatis* genetic

background is highly unfavorable; potentially due to the presence of a recombination “hotspot” occurring upstream of *TC0440*. It may also be the result of more complicated outcomes, such as toxicity to *C. trachomatis*, incompatibility with cellular machinery, or the requirement of other genetic factors that may not have transferred with these respective loci. Similarly interesting is the number of samples found to possibly recombine at the same region of homology. It was found using whole genome sequencing that the first recombination crossover occurred intergenically between *trpA* and *TC0440* for three of the isolates: 3220, 3106, and 2043 (Figure 2.6). Nearly all-remaining samples amplified sequence for both *trpA* and *TC0440*, suggesting the potential for crossover at this region as well. Collaborative efforts with Dr. Kevin Hybiske’s lab at the University of Washington revealed a number of recombinant clones from their library also show recombination at this exact sequence (personal communication, 2017). It is undetermined whether this region may be functioning as a chlamydial chi site since previous publications have analyzed recombination in *C. trachomatis* exclusively (Jeffrey, Suchland et al. 2013). It may be that this inter-species approach identified novel recombination “hotspots” within either *C. trachomatis* or *C. muridarum*. Another explanation for these observations may be directly related to the incapacity of *C. trachomatis* to incorporate the cytotoxins. As previously mentioned, efforts to clone them have been unsuccessful, theoretically due to their large ~10 kb size. This and other research may indicate size is not necessarily the limiting factor; rather, incorporating all three cytotoxins into *C. trachomatis* may be lethal. Within the isolates that did amplify cytotoxins, only two of the three were recombined, leading to the queries: 1) if it’s a viable possibility, why don’t more isolates show recombination incorporating these two cytotoxins and 2) why can’t *TC0437* transfer into *C. trachomatis*? It may simply be that sequence similarity flanking *TC0440* provides a more compatible region of homology. With the recent development

of both transformation and allelic exchange for use in *Chlamydia*, hypothesis such as this one can be readily examined.

This research describes the first evidence that homologous recombination may occur between *C. trachomatis* and *C. muridarum*. We also show that the incorporation of genes downstream of *TC0439* into *C. trachomatis* does not confer the ability to ascend in a mouse vaginal infection model; instead, it may result in a decreased ability to establish infection entirely, likely due to the loss of genes critical for growth and survival. It is anticipated that the ability to ascend is dependent on many genetic factors. Through recombination, large genome regions may be exchanged incorporating entire operons or gene clusters essential for pathogenesis. Recombination between these two species provides an opportunity to develop both *C. trachomatis* gain-of-function mutants capable of ascending to the UGT of mice, or loss-of-function *C. muridarum* isolates incapable of ascension. This development narrows the genome to easily discernible, phenotype-associated loci from which individualized genetic analysis can be performed. Subsequent identification of explicit factors essential to infectivity allows for generation of novel therapeutics targeting pathogen-specific markers. Conclusively, the innovation of this technique provides a fundamental approach for determining genetic correlates essential for infectivity and advances the study of this globally relevant human pathogen.

Chapter III.

Single Insertion and Disruption Using a Novel Transposon Mutagenesis

System for *Chlamydia* Identifies Genes Involved in Mammalian Infection

Abstract

Transposon mutagenesis is a widely applied and powerful genetic tool for the discovery of genes associated with selected phenotypes, including infection and pathogenesis. To apply this genetic tool for investigations in *Chlamydia*, a novel *HimarI* transposon mutagenesis system was developed. This system was used to generate two small libraries of transposon mutants: one for *C. trachomatis* and *C. muridarum*. The libraries include transposons inserted into genes encoding putative deubiquitinase (CT868/CTL0247; *cdu1*), membrane proteins (TC0411 and TC0189), competence associated (CT339/CTL0593; *comEC*) proteins as well as nine different proteins of unknown function. The majority of Tn insertion clones exhibited tissue culture growth characteristics similar to parental clones supporting that these gene products are not essential for *in vitro* growth. To determine if these insertional mutations have an effect during mammalian growth and infectivity, female mice were infected either transcervically (*C. trachomatis* mutants) or intravaginally (*C. muridarum* mutants). Four Tn mutants from *C. trachomatis* infected animals have shown significant decreases in recoverable organisms from the upper genital tract five days post-infection, whereas two from *C. muridarum* infected animals have shown similar decreases nine days post-infection. To date, this transposon mutagenesis system represents a novel technique for random, single-insertion disruptions in *Chlamydia* spp. and provides an innovative approach for elucidating specific genotype-phenotype correlations.

Chapter III.

Introduction

Transposon (Tn) mutagenesis is among the more effective strategies for discovering specific genetic components that are associated with a given phenotype. This genetic tool has been successfully applied for a better understanding of many basic biological processes as well as the discovery of gene products associated with host infection and pathogenesis in diverse Gram-negative and Gram-positive bacteria as well as *Mycobacterium* (Lampe, Akerley et al. 1999). For instance, evaluation of comprehensive transposon mutant libraries have led to the disruption, and ultimately the classification of genes responsible for *M. tuberculosis* growth within macrophages (Camacho, Ensergueix et al. 1999), tissue penetration and dissemination in *Pseudomonas aeruginosa* (Jacobs, Alwood et al. 2003, Liberati, Urbach et al. 2006, Alarcon, Evans et al. 2009) and *in vivo* bacteremia in *Streptococcus pneumoniae* (Hava and Camilli 2002). Through transposon-based discovery of virulence factors, many excellent candidates for directing novel antibiotics and therapeutic efforts have been identified.

Over a decade of optimizing naturally occurring transposon systems such as *Sleeping Beauty* and *Tc1/mariner* has resulted in a repertoire of successful genetic insertion systems (Hamer, DeZwaan et al. 2001). One of the more widely applied transposons is the hyperactive form of the *Himar1 mariner* system (Lampe, Akerley et al. 1999). This variant has resulted in robust transposon efficiencies and has revolutionized the study of genotype-phenotype correlation (Keravala, Liu et al. 2006). The *Himar1* system has many benefits that have allowed application in phylogenetically diverse bacteria (Finnegan 1992, Lampe, Churchill et al. 1996, Beare, Howe et al. 2009, Clark, Lackey et al. 2011, Cheng, Nair et al. 2013, Newton, Kohler et

al. 2014, Wood, Wood et al. 2014). The most important benefit is the simplified ‘cut-and-paste’ mechanism that requires a single transposase for recognition of cognate inverted repeat sequences on flanking ends of DNA. This allows for direct transposition of DNA to DNA, without the requirement of additional co-factors. Additionally, the *HimarI* hyperactive transposase has minimal target DNA specificity, inserting between A/T nucleobases (Benjamin and Kleckner 1992, Lampe, Churchill et al. 1996) and allowing for relatively non-specific insertion across an entire genome (Martinez and Delacruz 1990, Sota, Tsuda et al. 2007, Yahara, Didelot et al. 2014). The advantages of this system have proven to be beneficial for the study of pathogenesis in a diverse set of bacteria, including obligate intracellular organisms such as *Coxiella* and *Ehrlichia*. Research in *Coxiella* has demonstrated the use of *HimarI* Tn mutagenesis to identify genes critical for cellular growth and division *in vitro* (Beare, Howe et al. 2009, Newton, Kohler et al. 2014), whereas Tn studies in *Ehrlichia* revealed genes important for mammalian infection (Cheng, Nair et al. 2013). To date, however, a transposon mutagenesis system has not been developed for the globally prevalent and harmful obligate intracellular pathogen, *Chlamydia*.

Chlamydia trachomatis is the most commonly reported sexually transmitted bacterial infection in the United States and worldwide. *C. trachomatis* infections result in a range of health issues from pelvic inflammatory disease and sterility, to blindness or pneumonia (Hillis, Owens et al. 1997, Stephens 1999). *C. trachomatis* contains a single, circular chromosome of ~1.04Mb and a plasmid of ~7,500 bp (Stephens, Kalman et al. 1998, Thomson, Holden et al. 2008). This relatively small chromosome is predicted to contain approximately 900 coding sequences, yet ~35% of these encode for gene products lacking sufficient sequence similarity to support

functional annotation (i.e. hypothetical proteins) (Stephens, Kalman et al. 1998, Carlson, Porcella et al. 2005).

In order to study *in vivo* pathogenesis of this important human-adapted organism, the mouse-adapted *C. muridarum* has historically been employed. In mice, *C. trachomatis* establishes poor infections and lacks ascension to the upper genital tract, as is common with human female infections, whereas *C. muridarum* readily ascends to the upper genital tract and establishes many of the post-infection physiognomies observed in human pathology. *C. trachomatis* and *C. muridarum* share nearly 98% of their genetic makeup which allowed for interchangeability during a mouse infection model of pathogenesis in the past (Stephens, Myers et al. 2009). Research has since identified that the majority of genetic variability between the two species occurs within the plasticity zone (PZ)—a region where many genes associated with tissue tropism, host speciation, and infectivity are believed to be located (Rajaram, Giebel et al. 2015). For example, within the PZ of *C. trachomatis* are genes required for tryptophan synthesis that may account for tissue tropism, overcoming the host IFN-gamma response's depletion of intracellular tryptophan levels; whereas mice lack this IFN response, *C. muridarum* lacks this *trp* operon. Similarly, the *C. muridarum* PZ contains three large cytotoxins of unknown function, hypothesized to play a role in ascension in the mouse vaginal-infection model; two of these are deleted from the *C. trachomatis* PZ and the remaining cytotoxin is fragmented, assumed to produce no functional gene product (Rajaram, Giebel et al. 2015). It is hypothesized that these genetic differences within the PZ prevent interchangeability of these species, especially when analyzing correlates contributing to different pathologies during mammalian infections.

Enabled by the discovery of a method for transformation and effective selectable antibiotic markers (Wang, Kahane et al. 2011), there has been a recent surge of molecular tools

and methods developed for genetic manipulation in *Chlamydia* (Johnson and Fisher 2013, Wickstrum, Sammons et al. 2013, Bastidas and Valdivia 2016, Hooppaw and Fisher 2016). TargetTron (Johnson and Fisher 2013) and allele specific recombination (Binet and Maurelli 2009, Mueller, Wolf et al. 2016) are two tools that have been developed for targeted gene disruption and have enormous potential for functional studies and phenotype associations of candidate genes. However, for random mutagenesis and unbiased discovery strategies, only chemically induced mutagenesis has been developed (Kari, Goheen et al. 2011, Nguyen and Valdivia 2014, Kokes, Dunn et al. 2015). This strategy has been effectively employed to introduce multiple base mutations, including non-sense mutations, supporting an association of many gene products with noteworthy alterations of physiological pathways (Kokes, Dunn et al. 2015). While chemical mutagenesis is effective, certain limitations are associated, including the acquisition of multiple mutations. This can diminish the ability to correlate a phenotype to a particular genetic disruption. Furthermore, identifying the mutations requires whole-genome sequencing and revertants or compensatory mutations may be acquired during continued passaging. Thus, the development of a random single-insertion system, such as *HimarI* transposon containing a selectable marker, would allow for a straightforward generation of stable single site mutations, a simplistic identification of insertion sites, and the feasibility to screen for associated phenotypes.

Herein, we describe the development and application of a novel *HimarI* Tn system for *C. trachomatis* and *C. muridarum*. Through this innovative mutagenesis technique, the hyperactive C9 *HimarI* transposase was demonstrated to be functional, successfully inserting either the β -lactamase *bla* gene (*C. trachomatis*) or the chloramphenicol resistant *cat* gene (*C. muridarum*) into the respective chromosomes and conferring resistance. Relatively small libraries of Tn

insertion mutant strains were generated demonstrating that numerous coding regions were disrupted. Most insertion mutants exhibited a phenotype similar to wild-type, indicating that these gene products are not critical for *in vitro* cell-culture growth. Between the two libraries, four disruption mutants were found to have a role in establishing infections in mice transcervically infected with *C. trachomatis*, while two disruption mutants from the *C. muridarum* library displayed a decrease in recoverable organisms after a vaginal infection. These libraries offer a number of highlights to the field including identification of gene products essential for mammalian infection, potentially detecting species-specific mechanisms for infectivity, and distinguishing targets for directed mutagenesis in order to assess comparable phenotypes across many chlamydial species.

Chapter III.

Methods and Materials

Chlamydial strains and propagation. *C. trachomatis* serovar L2 434/Bu and *C. muridarum* Nigg were propagated from L929 mouse fibroblast cells (ATCC CCL-1) using RPMI medium 1640 (Invitrogen, Grand Island, NY) supplemented with 5% Heat-inactivated FBS plus 10 μ g/mL gentamycin (Fisher Scientific, Pittsburg PA). Briefly, L929 monolayers were grown to confluency in spinner flasks ($\sim 8 \times 10^5$ cells/mL) and infected with purified EBs. Cultures were allowed to grow for up to 48 hours at 37°C, 5% CO₂. Percent infectivity was assayed by IFA microscopy (described below). EBs were harvested as previously described (Mukhopadhyay, Clark et al. 2004) using a series of centrifugation and sonication steps to disrupt the host-cells and release EBs. Once isolated, EBs were either frozen immediately or further purified using Omnipaque™ (Barrington, IL) and ultracentrifugation. All EBs were stored in sucrose phosphate glutamate (SPG) media at -80°C. Clonal isolates were obtained as previously described (Suchland, Bourillon et al. 2005).

Development of pCMA and pCMC5M transformation plasmids. pUC19 (GenBank Accession # L09137) was used as the initial backbone for generating pCMA. The C9 hyperactive transposase was amplified from pBADC9 (kind gift from D. Lampe; Duquesne University (Lampe, Akerley et al. 1999)) and cloned between the EcoRI and AatII sites. An EagI site was incorporated for subsequent promoter cloning. Initially, a vector (pCMT) that contained a tetracycline encoding transposon was generated for application in *C. muridarum* using pACYC184 (GenBank X06403) as a template. Primers incorporated *Himar* inverted repeats and restriction sites for cloning into

the XmaI site. To remove the β -lactamase gene encoded on pUC19, the transposase, tetracycline transposon, and ori region were amplified with primers containing NcoI sites for self-ligation. Ligase independent cloning and HindIII sites were used to replace the tetracycline transposon with one encoding the β -lactamase and associated promoter from pSW2 (Wang, Kahane et al. 2011). To assist with selective expression of the transposase within *Chlamydia*, and less during plasmid propagation in *E. coli*, the chlamydial CT559 promoter and ribosomal binding sites were cloned upstream of the transposase gene using the EagI site (Hefty and Stephens 2007). pCMC5M plasmid was generated in which chloramphenicol resistance (*cam^R*) replaced the β -lactamase gene. Graphical plasmid depictions (Figure. 3.1) were generated using Savvy Scalable vector graphics (Bond and Naus 2012).

Transposon mutagenesis. Transformation of *C. trachomatis* was modified from previously described methods (Wang, Kahane et al. 2011). L929 cells were seeded to confluency in 6-, 12- or 24-well plates and allowed to adhere overnight. EBs and pCMA plasmid DNA were combined in CaCl₂ buffer to an MOI of 5 and incubated for 30 minutes at room temperature (RT). The mixture was added to 1X SPG before being overlaid on the L929 monolayer. Plates were spun at 550xg for 30 minutes at RT. Fresh RPMI supplemented with FBS, gentamycin and 1 μ g/mL cyclohexamide was added and plates were incubated at 37°C, 5% CO₂ overnight. 1 μ g/mL ampicillin (Fisher Scientific, Pittsburg, PA) was then added for antibiotic selection at 16 hours post-infection. Cultures were kept under antibiotic selection and serially passaged every ~36 hpi. 48 hrs after the final passage, EBs were harvested for identification of insertion site and subsequent analyses. *C. muridarum* transposon mutants were generated in the Hybiske lab at the University of Washington and were kindly sent for *in vivo* analysis.

Arbitrary PCR and confirmation of insertion sites. The Tn insertion site was determined using modified arbitrary PCR as previously described (Caetano-Anolles 1993, O'Toole, Pratt et al. 1999). Briefly, two rounds of PCR were performed using Taq polymerase. The first round was performed as follows: PCR along with round 1 ARB PCR primers (Table 3.1) with an annealing temp of 30°C for 6 cycles and 50°C for 30 cycles. PCR product was purified using a QIAquick clean up kit (Qiagen, Valencia, CA). The resulting product was used in the second round of PCR using round 2 ARB PCR primers and standard protocol. The final product was run on a 1.5% agarose gel and the resulting bands excised and sequenced. Sequences matching potential insertion sites were subsequently confirmed using PCR with primers designed to flank the suspected insertion site (Table 3.1). These amplicons were also run on a 1.5% agarose gel and the subsequent bands were sequenced to confirm the Tn insertion.

Digital Droplet PCR to identify single Tn insertions. Digital droplet PCR (ddPCR) was utilized as a highly sensitive method for determining the number of insertion sites of the transposon within the chromosome and was performed as per the manufacturer's instructions (Bio-rad, California). Mutant strains were harvested as described above and DNA was isolated and purified using the Qiagen Blood and Tissue kit (Qiagen, California). Primers were designed for the *β-lactamase* insertion sequences as well as 16S rRNA and *tsp* (*CT441*, Table 3.2). PCR Reactions were run using either EvaGreen® in an oil-in-water droplet emulsion generated using the droplet generator cassette (Bio-rad, California). The PCR conditions are as follows: 95°C for

Table 3.1: Primers used to identify and confirm Tn insert.

Transposon Flanking PCR (5'-3')	
<u>Gene</u>	<u>Primer Sequence and Designation</u>
Round 1 ARB primers	CTATGGATGAACGAAATAGACAG GGCCACGCGTCGACTAGTACNNNNNNNNNGATAT
Round 2 ARB primers	CACTGATTAAGCATTGGTAACTGTC GGCCACGCGTCGACTAGTAC
<i>CT015</i>	CGATATACATATACCGGGTGTTCAG GTGTTAACCGGCGATCCTAC
<i>CT036</i>	GGGAGCGAGTTTCTTAGTTCTC TACAGACTGGACACCACAAATC
<i>CT148</i>	GAAGAAAGAGAGCCAGAGGTATTT GGTGACCCATTTACTCGAAGAG
<i>CT153</i>	CGTCTTTAGTTGTCTCCCAAGA GCCTTCTTTCTTATACGTTATTCAACC
<i>CT333</i>	TCCTTCACCGATCTATCCTCTT TGACATTGGTTACACGGAGTTT
<i>CT339</i>	TGCCATCTTCCTTATGATTCC TGGTAGCAGAGAGAGCTTACA
<i>CT404</i>	CCTGAAGGCAACCGTTGAT AGGTCCTGATGGAGAGACATT
<i>CT550</i>	AGAACTCTTCACCCTGGTTTA GAAGCGGAAGAGACCGGTGGAACCTGCTGATGGTCTGCT
<i>CT696</i>	GGTGCAACACCACATTCATTTA CGGAAAGTTCCATCTCCTGATT
<i>CT819</i>	TGCCAGGGACCTTCTCTT CCATTACAGCAGGGACAGAAA
<i>CT868</i>	CCTCCTTTGCTGCAATCTTTAC CTCGGATACCATGCAGACTATG
<i>CT383/384 IGR</i>	GCAATAGCCTTCTATTTAGGAGC GATTTTGTGTAACCATTACAAGAAGAG
Tn Inverted Repeats	GGCTGATAAGTCCCCGGTC GGCTGATAAGTCCCCGGTC

10 minutes, followed by 40 cycles of 94°C for 30 seconds, and 60°C for 1 minute. Finally, reactions were held at 98°C for 10 minutes followed by cooling to 4°C. Once complete, fluorescence of individual droplets was calculated using a droplet reader (Bio-rad, California) and analyzed using the QuantaSoft Software (Bio-rad, California). Data is reported as a ratio of copies/ μ L for respective primer comparisons.

Immunofluorescence microscopy. For determination of percent infectivity, calculating infective titer and phenotypic analysis, IFM was performed as previously described (Osaka, Hills et al. 2012). L929 cells were grown to confluency in an 8-well ibiTreat μ -Slide (Ibidi, Martinsried, Germany) and were infected with respective *C. trachomatis*/Tn mutants. At ~24 hpi, infected cells were fixed with 100% methanol for 10 minutes at RT. Cells were washed once with HBSS and again with PBS then stained using either 180 μ l of the MicroTrack *C. trachomatis* culture confirmation test (Syva Co., Palo Alto, CA) or Virostat *Chlamydia* MONOTOPE Fluorescein Conjugated IgG1 LPS, diluted 1:40 in PBS for 1 hour and 50 minutes RT in the dark or overnight at 4°C. 20 μ l of 1 μ M 4', 6-diamidino-2-phenylindole (DAPI) diluted 1:100 in PBS was then added to wells and allowed to stain for 10 minutes, RT in the dark. Stain was then removed, and the cells washed with PBS. For *in vitro* phenotypic analysis, a final overlay of Vectashield antifade mounting medium (Burlingame, CA) was added and slides were stored at 4°C in the dark until imaged. Cells were visualized on an Olympus IX81/3I spinning disk confocal inverted microscope at 150X magnification and captured on an Andor Zyla 4.2 sCMOS camera (Belfast, Northern Ireland). Microscope and camera were operated using SlideBook 6 software (Intelligent Imaging Innovations, Denver, USA). Exposure time remained consistent for all fields captured, with exposure for DAPI at 2 seconds, MOMP 4 seconds, and cytoplasm 6 seconds. Three to

Table 3.2. Primers used for Droplet Digital PCR

Droplet Digital PCR (ddPCR) (5'-3')	
<u>Gene</u>	<u>Primer Sequence and Designation</u>
β-lactamase (transposon)	TTGGTAGCTCAGAGAACCTTC CTTGAGATCGTTTTGGTCTGCG
<i>16S</i>	GCAACGAGCGCAACCCTTATC TTGACGTCATCCTCGCCTTCC
<i>tsp</i>	TGTGTGGCATAGGAGTCGTGC CGTTTTATCAGCAGGGCCTCC
<i>C. trachomatis secY</i>	TAAAAAGCCGTGTCATTTCGTCC TCGGCTTCAATCATTGTACAGC
<i>C. trachomatis secY</i> probe	/56-FAM/TAATTTACG/ZEN/CTTCCCTTGATCCGGC/3IABkFQ/ CAGATGGGAGGACARAGATTCGGGG
<i>C. muridarum rpoB:</i>	TGAACGACTCAGGCGTTCCAGAAC
<i>C. muridarum rpoB</i> probe	/56-FAM/CGATGTTTC/ZEN/GGGAAGAACTCGT/3IABkFQ/ CTCTTCCAGTGTGCAAGAAAGC
Murine <i>rpp30</i>	AGTGACTGATGAGCTACGAAGG
Murine <i>rpp30</i> probe	/5HEX/TGAGACGAGTCCTGAGTCTC/3IABkFQ/

seven Z stack images at 0.3 μ m apart were taken per cell imaged. Images were processed in SlideBook 6 and a No Neighbors Deconvolution with a subtraction constant of 0.4 was applied to all images. For calculations of infective titers, a final overlay of 0.1M tris-glycerol was added and cells were imaged using an Olympus IX81 inverted epifluorescence microscope. For each Tn mutant, IFU/ μ L was calculated and used as the pre-infection titers.

In vivo mouse infections. Female C57BL/6 mice 6-8 weeks old were purchased from Jackson Laboratories and housed in accordance with the University of Kansas Institutional Care and Use Committee requirements. Mice were treated subcutaneously with 2.5mg Medroxyprogesteronacetat (Depo-Provera, Pfizer, NY) upon arrival (day -7). Fresh aliquots of WT *C. trachomatis* or *C. trachomatis* Tn mutant stocks were diluted in sucrose-phosphate glutamic-acid (SPG) buffer and kept on ice until use. Using a non-surgical embryo transfer device (NSET, Lexington, KY), mice were inoculated transcervically with 10 μ L diluted stock (final concentration $\sim 5 \times 10^5$ IFU/mouse) by inserting the device into the genital tract beyond the opening of the cervix. Mice were monitored to ensure no dose was secreted from the animal. For *C. muridarum* vaginal infections, WT or Tn mutants were diluted to a final dose of 1×10^5 IFU/5 μ L in SPG and kept on ice until use. Animals were swabbed using sterile nasopharyngeal swabs (Puritan, Guilford, Maine) prior to dosing. Using a P10 pipette, sterile tips were inserted until reaching the cervix of the animals, slightly withdrawn and inoculated. Animals were held briefly with the tip still inserted to ensure no dose was secreted or pulled out upon removal of the pipette tip. Post-infection titers were calculated for each dose by infecting L929 monolayers with serial dilutions of each dose. Staining and IFU/ μ L calculations were performed as described above.

ddPCR assessment of *Chlamydia* infection *in vivo*. Infected mice were humanely euthanized five days post-infection (*C. trachomatis* transcervical infections) or nine days post-infection (*C. muridarum* vaginal infection) and the genital tracts were collected in SPG. Organs were homogenized using a rotor/stator homogenizer (Biospec, Bartlesville, OK). 100uL aliquots were frozen at -20°C until use. Remaining stocks of homogenized tissues were stored at -80°C. DNA isolation on the 100uL aliquots was performed using the DNA isolation using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Isolated DNA was then used to run droplet digital PCR similarly as described above. Primers and probes for *C. trachomatis secY*, *C. muridarum rpoB* and mouse *rpp30* can be found in Table 3.2. ddPCR Supermix for Probes was used to set up PCR reactions and Oil-for-Probes droplet emulsions were generated using the droplet generator cassette (Bio-rad, California). The PCR conditions are as follows: 95°C for 10 minutes, 40 cycles of 94°C for 30 seconds, and 98°C for 10 mins followed by cooling to 4°C. Fluorescent reads of individual droplets were calculated after PCR using the QX200 droplet reader (Bio-rad, California). Data was analyzed using the QuantaSoft Software (Bio-rad, California) and reported as a ratio of *Chlamydia* DNA to host DNA (*secY* or *rpoB/rpp30*, copies/ μ L). Box and whisker scatter plots were generated in Graphpad Prism 7 and recoverable organisms were compared using unpaired, multiple t-tests with no correction for multiple comparisons.

Chapter III.

Results

Development of transposon plasmid.

Typical transposon mutagenesis strategies include the introduction of two plasmids: one encoding the transposase and another containing the transposon. The intent of keeping these components separate is to reduce the acquisition of deleterious mutations in the transposase or transposon during plasmid propagation in *Escherichia coli*. Due to the very low transformation efficiencies in *Chlamydia* (Beare, Sandoz et al. 2011, Bastidas and Valdivia 2016, Hooppaw and Fisher 2016), it was necessary to design a plasmid that contained both components, yet limit the expression of the transposase in *E. coli*. The transposon plasmids, termed pCMA and pCMC (Chlamydia Mariner and Chlamydia Mariner Chloramphenicol plasmids, Figure 3.1) were designed to encode the widely utilized C9 *Himar1* transposase. To assist with selective expression of the transposase within *Chlamydia*, and less during plasmid propagation in *E. coli*, the chlamydial CT559 promoter and ribosomal binding sites were cloned upstream of the C9 transposase. This promoter demonstrated activity in *Chlamydia*, but limited transcriptional activity in *E. coli* (Wickstrum, Sammons et al. 2013). pCMA also contained the β -lactamase encoding gene preceded by regulatory components previously demonstrated to be effective in the chlamydial shuttle vector pSW2 (Wang, Kahane et al. 2011), whereas pCMC contained the GFP-fused chloramphenicol resistance gene. Both antibiotic resistance genes were cloned with flanking inverted repeats for recognition and transposition by the *Himar1* transposase. Additionally, the *colE1* origin of replication from *E. coli* was used to enable propagation in *E. coli*, but no replication in *Chlamydia*, resulting in a replication incompetent suicide vector.

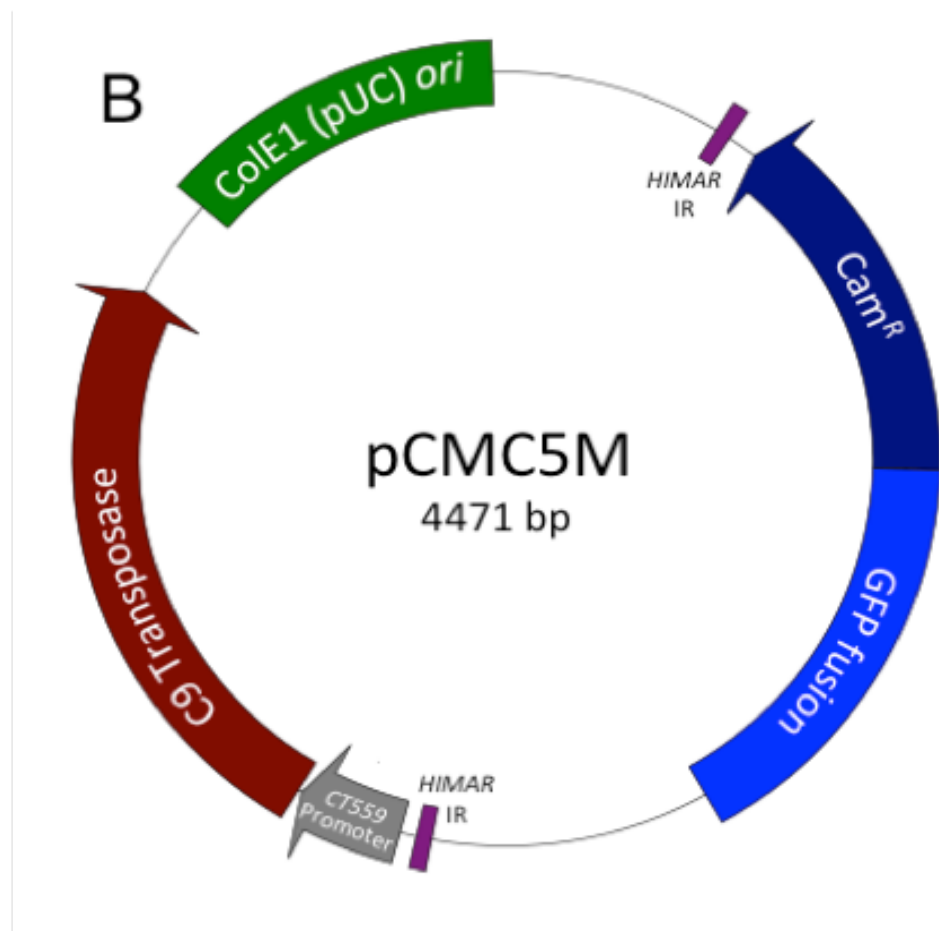
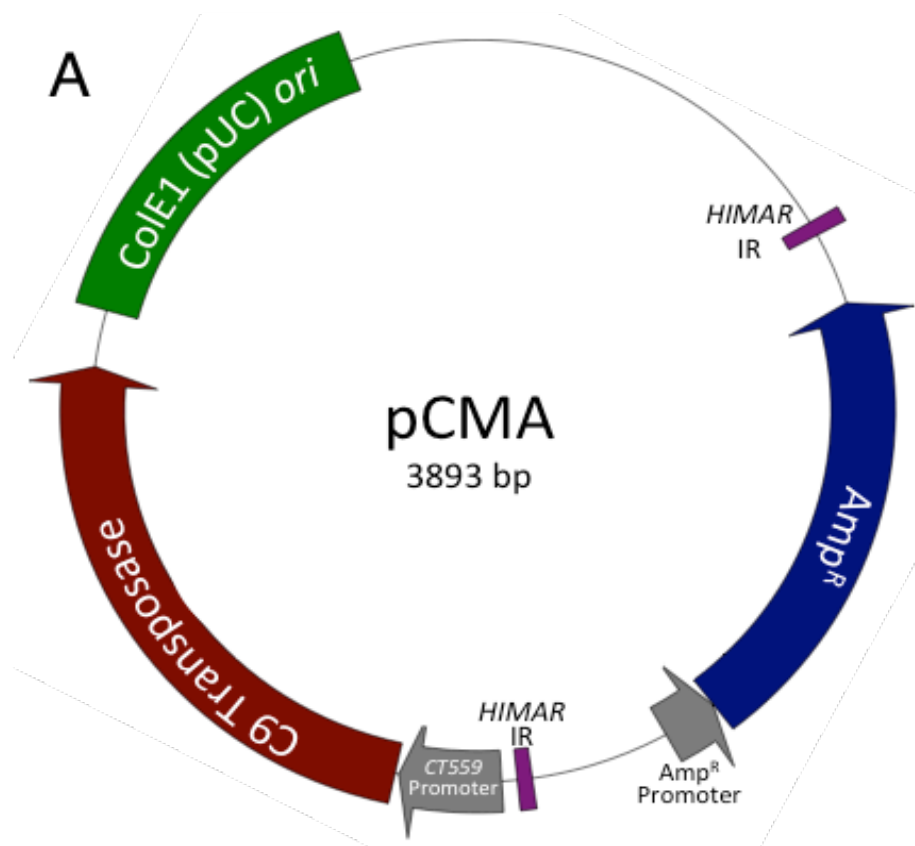


Figure 3.1 Plasmid maps of pCMA and pCMC5M. The C9 hyperactive variant of the *Himar1* transposon was cloned downstream of the *Chlamydia* CT559 promoter. A) β -*lactamase* and associated promoter was cloned from the chlamydial shuttle vector pGFP::SW2 (Wickstrum, Sammons et al. 2013). B) GFP-fused chloramphenicol resistance replaces the β -*lactamase* and promoter. Both antibiotic markers are flanked by *Himar1* inverted repeats to generate the transposon.

Transposon mutagenesis with pCMA yields stable Tn insertion mutants.

The pCMA plasmid was used in a standard transformation procedure using β -lactams for selection in *C. trachomatis*. This procedure typically requires three passages under antibiotic selection (infection-growth-lysis-reinfection) to ensure that resulting organisms contain β -lactamase coding DNA. Clonal isolates from this resistant culture were obtained and expanded. PCR confirmed the presence of the β -lactamase gene, yet PCR was negative for the presence of the transposase (data not shown) supporting the loss of the non-replicative pCMA plasmid. To identify the site of potential transposon insertion, arbitrary PCR (O'Toole, Pratt et al. 1999) was performed and sequence analysis of individual amplicons indicated the presence of the β -lactamase transposon. Subsequent PCR with primers flanking the possible transposon insert supported the insertion of ~1400 bp (Figure 3.2) Moreover, only this larger amplicon size was observed, supporting the clonality of this sample (i.e. no wild-type or alternate Tn insertion mutants present). Sequencing of the amplicons confirmed the presence and provided the exact location of the transposon insertion between a TA for each insertion site (Figure 3.3.). Similar arbitrary PCRs were performed on *C. muridarum* Tn mutants using the pCMC5M plasmid and exact location of the transposon insertion was identified (Figure 3.4) For both libraries, whole-genome sequencing was also performed to confirm singularity and location of Tn insertion (data not shown).

In order to decrease the potential for normal growing mutants to outcompete fitness compromised mutant clones in a mixed infection, as well as to evaluate transformation and transposon efficiency, two transformation reactions (DNA and EBs) were mixed and incubated before being split into individual 12-well plates. Each well was passaged twice with selection and after the second passage; cultures were allowed to continue with daily monitoring of

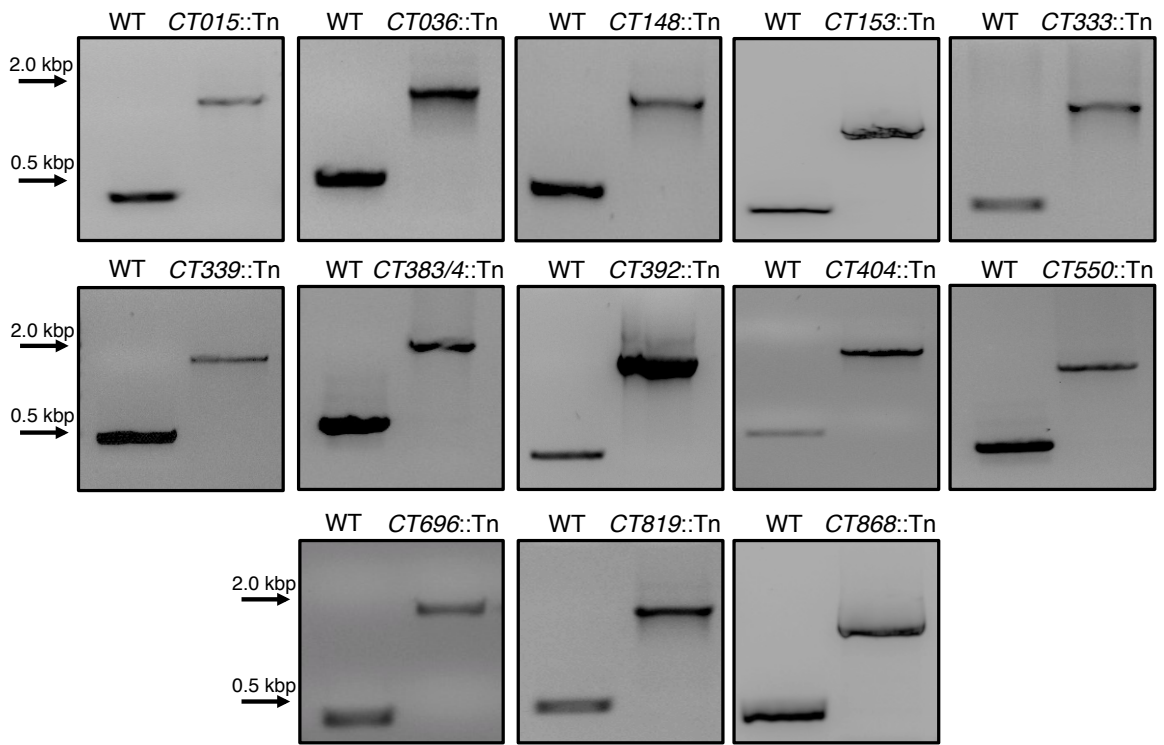


Figure 3.2. PCR confirmation of Tn insertion location. DNA was extracted from each *C. trachomatis* Tn mutant and analyzed by PCR using primers flanking the proposed Tn insertion sequence. A single band of ~2.0 kbp was observed indicating the presence of the Tn with flanking gene overhangs (size of WT gene, ~0.5kbp and Tn insertion sequence, ~1.4 kbp). Absence of lower native gene band supports clonal isolation of cultures. See table 2 for primer descriptions.

C. trachomatis growth. Within 3 days of cultivation, *C. trachomatis* growth was observed in 5 or 6 wells of separate 12 well plates. Growth of one sample (*CT696::Tn*) took 8 days before growth was observed and required addition to fresh monolayers to allow continued propagation. These data support that transformation efficiency in *C. trachomatis* is very low (estimated <1 IFU/ μ g DNA) as well as the possibility that *C. trachomatis* is less tolerant to disruptive insertions. These data also support that growth observed in each of these wells is likely clonal.

While arbitrary and flanking PCRs indicated the presence of a single transposon insertion in *C. trachomatis* mutants, it appeared several *C. muridarum* Tn mutants potentially had double inserts. Due to the ease and accessibility of our collaborators to whole genome sequencing (WGS), follow-up sequencing on many *C. muridarum* Tn mutants further identified four isolates with double insertions. For one isolate, only one copy of the Tn (chloramphenicol) gene is present, however, the position upon which it inserted resulted in a disruption of two overlapping genes: *TC0368* and *TC0369* (Figure 3.5A). In our laboratory, to evaluate the possibility of additional transposon insertions, droplet digital PCR was implemented. Droplet digital PCR (ddPCR) is a relatively new technology, which allows for accurate quantification of the copy number for individual alleles (Roberts, Last et al. 2013). It also requires very little sample relative to other methods. ddPCR was initially performed on *CT392::Tn* and wild-type *C. trachomatis*. *C. trachomatis* encodes for two copies of *16S* rRNA, providing an internal control for the detection of multiple alleles, and tail-specific protease (*tsp*) was used as a single-copy allele control. As Figure 3.5B indicates, allele quantification of *16S* and *tsp* for wild-type and *CT392::Tn* resulted in *16S/tsp* allele copy ratios near 2. However, when β -lactamase (*bla*) allele copies were enumerated and compared to *tsp*, these ratios were near 1, supporting the presence of a single insertion of the transposon. This analysis was performed for the remainder of the

A

CT015::Tn (*phoH*-Phosphate Responsive ATPase)



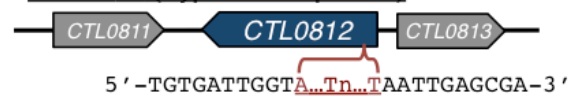
CT404::Tn (SAM dependent methyltransferase)



CT036::Tn (*Hyp*¹-Putative exported protein)



CT550::Tn (Hypothetical protein)



CT148::Tn (*mphA*-FAD-Dependent monooxygenase)



CT696::Tn (Hypothetical protein)



CT153::Tn (*macP*-Membrane attack complex)



CT819::Tn (*yccA*-BAX inhibitor)



CT333::Tn (*uvrA*-NER endonuclease)



CT868::Tn (*dub1*-Deubiquitinase)



CT339::Tn (*comEC*-Competence DNA uptake)



CT383/384::Tn (Intergenic region)



CT392::Tn (Hypothetical protein)



B

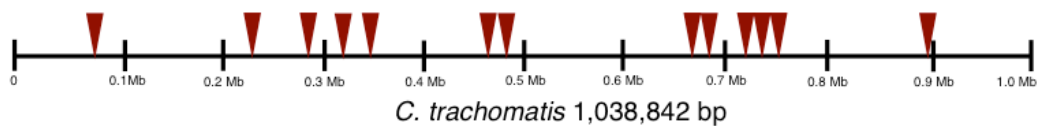


Figure 3.3. *Himar1* transposon insertions sites for *Chlamydia trachomatis* mutants. A) Both *C. trachomatis* serovar D (CT) and LGV (CTL) gene nomenclature indicating site of Tn insertion and immediate flanking genes (gray) are shown. Precise insertion of transposon (red) and flanking sequence (black) including the A/T upon which the transposon inserted are highlighted. B) Diagram showing relative insertions (red) within the *Chlamydia* genome indicating the dispersion of Tn insertions.

transposon mutant clones revealing that all samples had *bla* to *tsp* ratios close to 1 (range 0.7 – 1.15; Figure 3.5C), also supporting the presence of a single transposon insertion in each clone.

In total, 13 unique *C. trachomatis* Tn mutants and 26 *C. muridarum* Tn mutants were generated within a variety of genetic targets (Figures 3.3 and 3.4). Within the *C. trachomatis* library, all but one (intergenic region *CT383/4::Tn*) of these transposon insertions occurred within a coding region, likely disrupting the function of the encoded gene product. Moreover, the insertions are occurring throughout the genome with slight enrichment of 5 Tn insertions within ~100 kb near 0.7 Mb site (Figure 3.3B). Four Tn insertions occurred within genes encoding hypothetical proteins: CT036, CT392, CT550, and CT696. Among functionally annotated genes, insertions occurred in a range of genes including those encoding a phosphate responsive ATPase (CT015), FAD-dependent monooxygenase (CT148), MAC/Perforin family protein (CT153), DNA repair mechanisms (CT333), competence factor (CT339), SAM-dependent methyltransferases (CT404), apoptosis inhibitor (CT819), and deubiquitinase (CT868).

Within the *C. muridarum* Tn library, all but four isolates resulted in insertions within coding regions. Seven of these occurred within hypothetical proteins, including the same deubiquitinase observed to contain an insert within the *C. trachomatis* library (*CT868/TC0259*). Among isolates containing double inserts, a region spanning >150kbp is present between each insert site and two of these isolates contain insertions disrupting genes within the plasticity zone: a region of high variability hypothesized to be the determinant for virulence and host-tropism. Further, three inserts disrupted polymorphic membrane proteins: surface exposed proteins necessary for adhesion to host epithelial cells and virulence of the organism (Becker and Hegemann 2014).

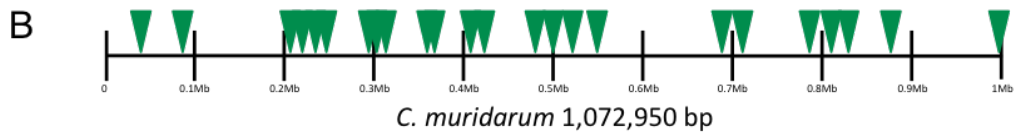
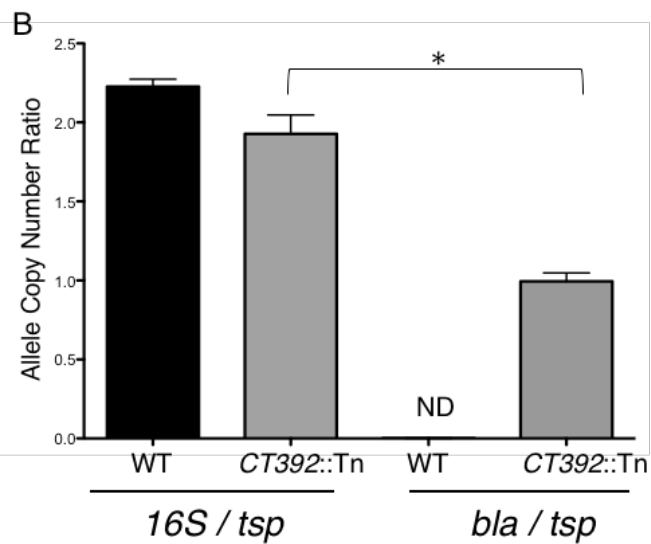
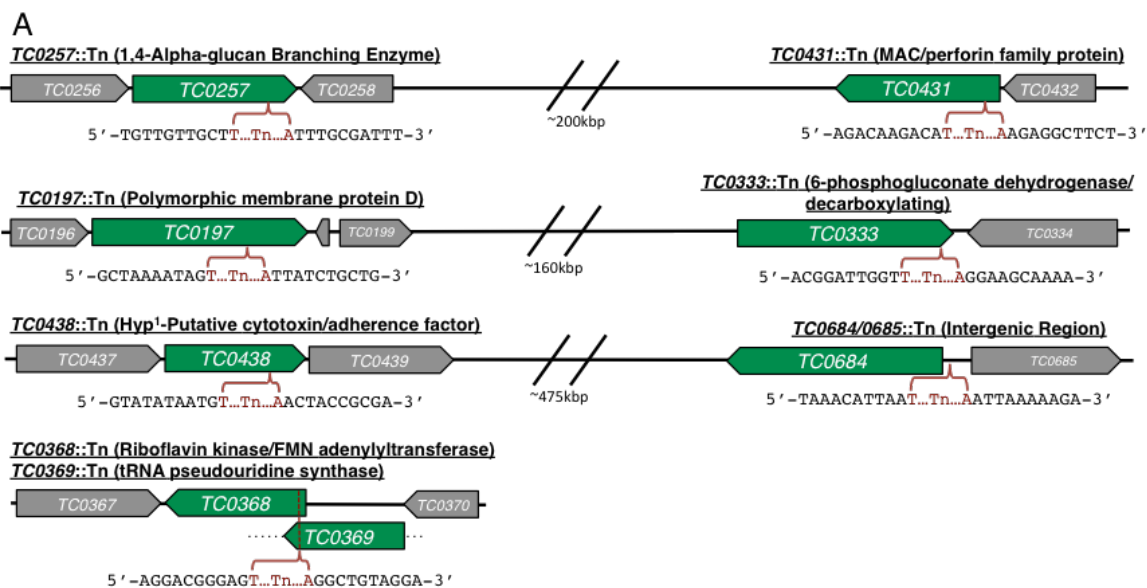


Figure 3.4. *Himar1* transposon insertions sites for *Chlamydia muridarum* mutants. A) *C. muridarum* Nigg gene nomenclature indicating site of Tn insertion and immediate flanking genes (gray) are shown. Precise insertion of transposon (red) and flanking sequence (black) including the A/T upon which the transposon inserted are highlighted. B) Diagram showing relative insertions (green) within the *Chlamydia* genome indicating the dispersion of Tn insertions.



C

Tn Mutant	Ratio <i>bla</i> : <i>tsp</i>
CT015::Tn	0.84
CT036::Tn	0.92
CT148::Tn	0.84
CT153::Tn	0.79
CT333::Tn	1.05
CT339::Tn	1.08
CT392::Tn	0.89
CT404::Tn	1.12
CT550::Tn	0.84
CT696::Tn	1.15
CT819::Tn	0.81
CT868::Tn	0.70
CT383/384::Tn IGR	0.77

Figure 3.5. Assessment of single vs. double Tn insertions. A) Whole genome sequencing of *C. muridarum* Tn mutants confirmed the presence of double inserts in 4 isolates. Gene nomenclature indicating site of Tn insertion and immediate flanking genes (gray) are shown. Transposon (red) and flanking sequence (black) including the A/T upon which the transposon inserted are highlighted. Distance between genes containing inserts is shown as double-backslashes with the length between in kbp. *TC0368* and *TC0369* genes overlap, resulting in a double insert at the same region (only 1 Tn). B) Ratio of allele copy numbers for *CT392::Tn*. Genomic DNA was isolated and subjected to ddPCR with primers specific for *tsp*, *16S*, and *bla*. Data are reported as mean and SD of allele copy number ratios. As expected, *bla* generated an equal one-to-one ratio with *tsp*, indicating the presence of only one Tn insertion per genome. The absence of endogenous *bla* is observed (ND: None detected). Statistical difference in measured ratio of *16S/tsp* to *bla/tsp* within *CT392::Tn* was determined by unpaired t-test (* $p=0.004$). C) Copy numbers for all *C. trachomatis* Tn mutants. Subsequent ddPCRs were performed on all remaining Tn mutants. Data are reported as allele copy number ratio of *bla/tsp*.

General growth characteristics of transposon mutants.

Following passaging and confirmation of transposon insertions, mutant *C. trachomatis* inclusions and bacterial cell morphology were imaged and assessed by confocal microscopy. For most of the transposon mutants, inclusion and bacterial morphology yielded no apparent abnormalities (Figure 3.6). A notable difference in growth and inclusion morphology was observed for the clone containing a transposon insertion in the gene encoding the hypothetical protein CT696. This mutant appears to have a severely reduced ability to grow and form inclusions relative to wild-type *C. trachomatis* (Figure 3.7). Typically, growth and division of reticulate bodies are evident (Figure 3.7A, WT and 3.7B, *CT696::Tn*; white arrows), however, RBs from *CT696::Tn* are dispersed throughout the cytoplasm and do not appear to localize near the nucleus as generally observed in early inclusions (Figure 3.7B and 3.7C). Occasionally, bacteria appear to aggregate (Figure 3.7D; yellow arrow), exhibiting abnormal patterns within an inclusion, however, individual RBs retaining their expected coccoid morphology are still observed (Figure 3.7D; blue arrow).

In vivo assessment of transposon infectivity and recoverable bacterial burden.

As initial studies have elucidated, mouse infections with *C. trachomatis* L2 administered vaginally are readily cleared, does not cause pathology and rarely ascends to the upper genital tract—the biologically relevant site for mimicking human infections (Gondek, Olive et al. 2012). As such, the transcervical model was developed in which infective doses of *C. trachomatis* L2 (434/Bu) are dispensed directly into the upper genital tract. These proof-of-concept experiments have shown that infections typically peak between days 3 and 6 post-infection and have cleared dramatically (nearly 3 log-fold) after ~9 days post infection (Gondek, Olive et al. 2012).

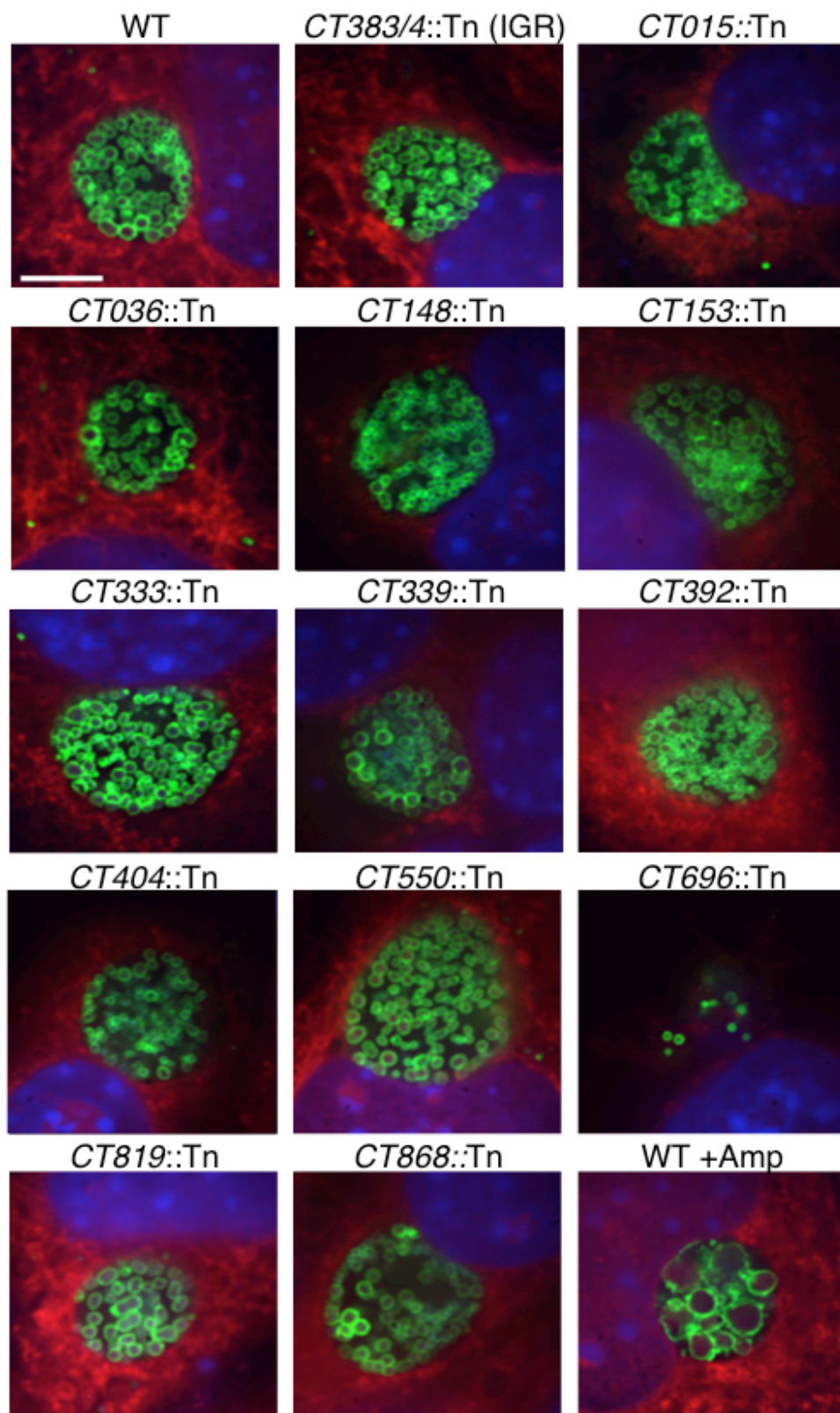


Figure 3.6. Confocal Microscopy of *C. trachomatis* Tn Mutants. L929 cells were infected with either WT *C. trachomatis* or respective Tn mutant. 24 hpi μ -slides were stained. Ampicillin (Amp) treated cells were included as a representative image of persistence found in non-transformed bacteria. Cells were viewed at 150X magnification with no Neighbors deconvolution and a subtraction of 0.4 in SlideBook. Blue: DAPI, nucleus; Red: Evan's Blue, cytoplasm; Green: MOMP, *C. trachomatis* organisms. Scale bar = 5 μ m.

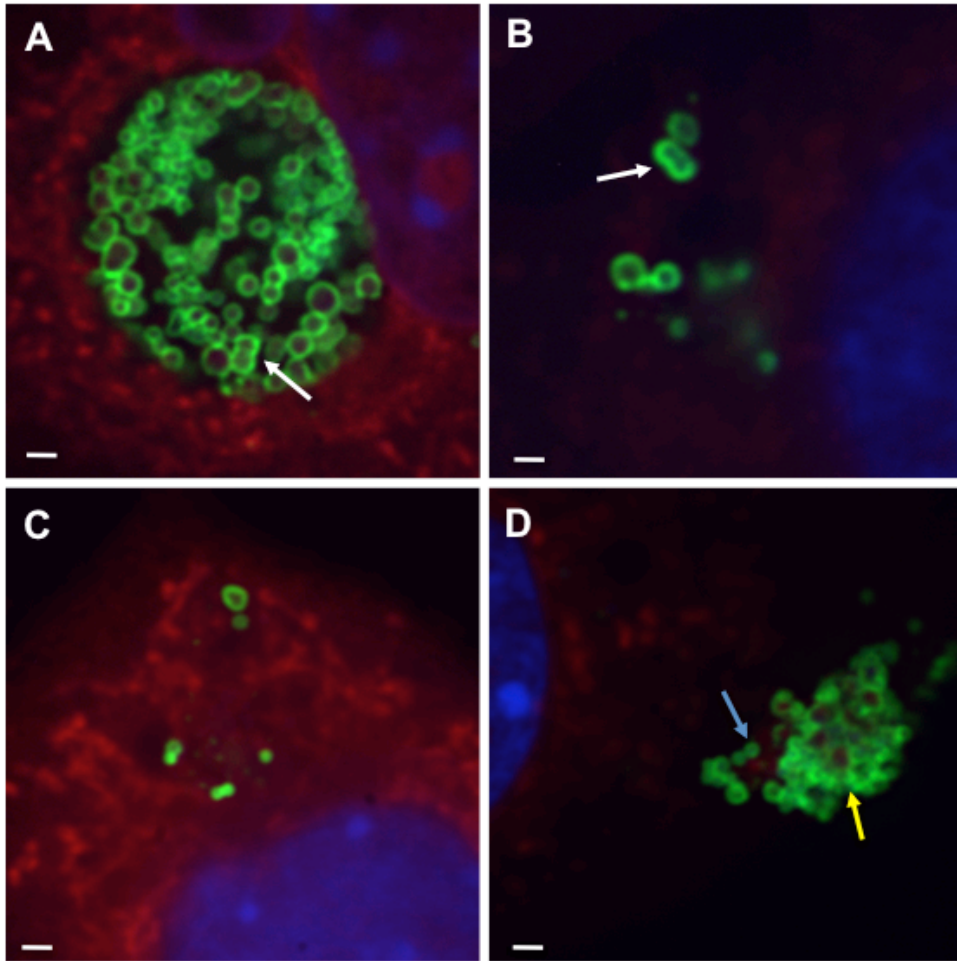


Figure 3.7. Confocal microscopy of *CT696::Tn* mutant. L929 mouse fibroblasts were infected with either *C. trachomatis* WT or *CT696::Tn* mutant. Infections were fixed at 24 hpi and immunofluorescently stained. Images were acquired at 150X magnification with deconvolution for enhanced image resolution. A) WT *C. trachomatis* inclusion showing normal RB division (white arrow). B) *CT696::Tn* showing normal RB division (white arrow). C) Dispersed localization of *CT696::Tn* within the cytosol. D) Aggregating form of *CT696::Tn*. Coccoid shaped RBs (blue arrow) are observed external to the aggregation (yellow arrow). Blue: DAPI, nucleus; Red: Evan's Blue, cytoplasm; Green: MOMP, *C. trachomatis* organisms. Scale bar = 1 μ m.

C. muridarum, on the other hand, is capable of ascending to the upper genital tract rapidly after vaginal infections, and remains detectable far beyond 15 days post infection (Gondek, Olive et al. 2012). Therefore, to illuminate potential deficiencies of Tn mutants to establish infection, we utilized both models in which genital tracts of *C. trachomatis* infected mice were harvested on day 5, within the peak of WT L2's dynamic range of infection, and *C. muridarum* infected mice were harvested on day nine post-infection. It is hypothesized the Tn insertion(s) within intergenic regions (*CT383/4::Tn*, *TC0019/20::Tn*, *TC0080/1::Tn*, *TC0436/7::Tn*, *TC0913/4::Tn*) may serve as an internal Tn-controls due to the lack of disruption within a coding region and, as such, any potential deficiencies due to the presence of the transposon itself will be observable using these mutants. Further, whole genome sequencing of the mutants confirmed any potential phenotypes observed are the result of Tn disruptions and not additional mutation incurred during propagation.

As can be seen from Figure 3.8, the *C. trachomatis* intergenic disruption (*CT383/4::Tn*) showed no significant difference in detectable organisms as compared to WT *C. trachomatis* infection and serves as an effective control for comparison between Tn insertion mutants. Of the remaining 11 Tn mutants, two showed statistically significant decreases in detectable organisms from the uterine horns whereas two showed a decrease, albeit not statistically significant, as compared to the intergenic Tn control. Disruptions in a hypothetical-putative exported protein (*CT036*), the membrane attack complex (*CT153*) and the ChlaDUB1 deubiquitinase (*CT868*) showed nearly a half- to log-fold decrease in detectable organisms as compared to IGR. An *mphA*-FAD-dependent monooxygenase (*CT148*) showed a range of infectivity with one animal completely uninfected and three exhibiting WT levels of detectable organisms (Figure 3.8). The mean detectable values for this group fall two-log below the means of both WT and IGR, the

most dramatic deficiency in detectable organisms. What remains unclear, however, is whether these Tn mutants deficient in infectivity are incapable of establishing infection altogether or whether the peak of infectivity is delayed; thusly future studies in which recoverable organisms are assessed at varying timepoints throughout an infection should be implemented. Similarly, infections using a higher IFU will provide strong support for the mutants' inability to establish infection outright.

Due to the consistent expansion of the *C. muridarum* library taking place at the Hybiske Lab, *in vivo* analyses remain ongoing. Preliminary data from seven of these Tn mutants, however, identified two potential candidates with deficiencies in mammalian infections: *TC0657::Tn* and a double-insert mutant *TC0257/TC0431::Tn* (Figure 3.9). Similar to *C. trachomatis* infections, the intergenic Tn mutant *TC0436/7::Tn* showed no significant difference in detectable organisms as compared to WT *C. muridarum*. A Tn insertion within the glucose-6-phosphate isomerase (*TC0657*) showed a significant defect in recoverable organisms from the uterine horns, however no other mutants showed similar deficiencies.

Perhaps the most intriguing of the *C. muridarum* Tn mouse infection data are the animals infected with the double-insert isolate, *TC0257/TC0431::Tn*. As can be seen in Figure 3.9, nearly all animals within this group display a defect in infectivity, with the respect of two animals. These two mice displayed hyper-infections with detectable organisms as high as the ovaries (data not shown). At the time of infection, it was not known that this isolate contained a double insert; rather it was presumed to only contain one or the other. To date, it still remains unclear whether the sample contains two inserts within one genome or a mixed population with single and double inserts; current efforts are underway to plaque isolates from the uterine horns of these animals to determine if they contain one or both disruptions. While it is known

Chlamydia accumulates glycogen for metabolism, the regulation of this metabolism remains elusive; recent studies have shown that even when grown in variable media, expression of many gluconeogenic genes remain constant (Iliffe-Lee and McClarty 2000) and thus, suggest a disruption in *TC0257 (glgB)* may have a dramatic effect in growth of the organisms. Equally, the chlamydial MACPF (*TC0431*) is a highly conserved protein within the plasticity zone which may serve as a potential virulence factor (Taylor, Nelson et al. 2010) that, if disrupted, may explain a dramatic decrease in the organism's ability to establish infection in the mouse model. To fully interpret the effect of these mutants the determination of double vs. single/mixed insertion mutants must be identified. Secondly, the animals displaying a decrease in recoverable organism should be assessed to determine if one or both insertion disruptions remain and finally, organisms from the two hyper-infected mice will need to be assessed for the potential of variable genotypes as compared to the remaining animals within this group: either through compensatory mutations or revertants.

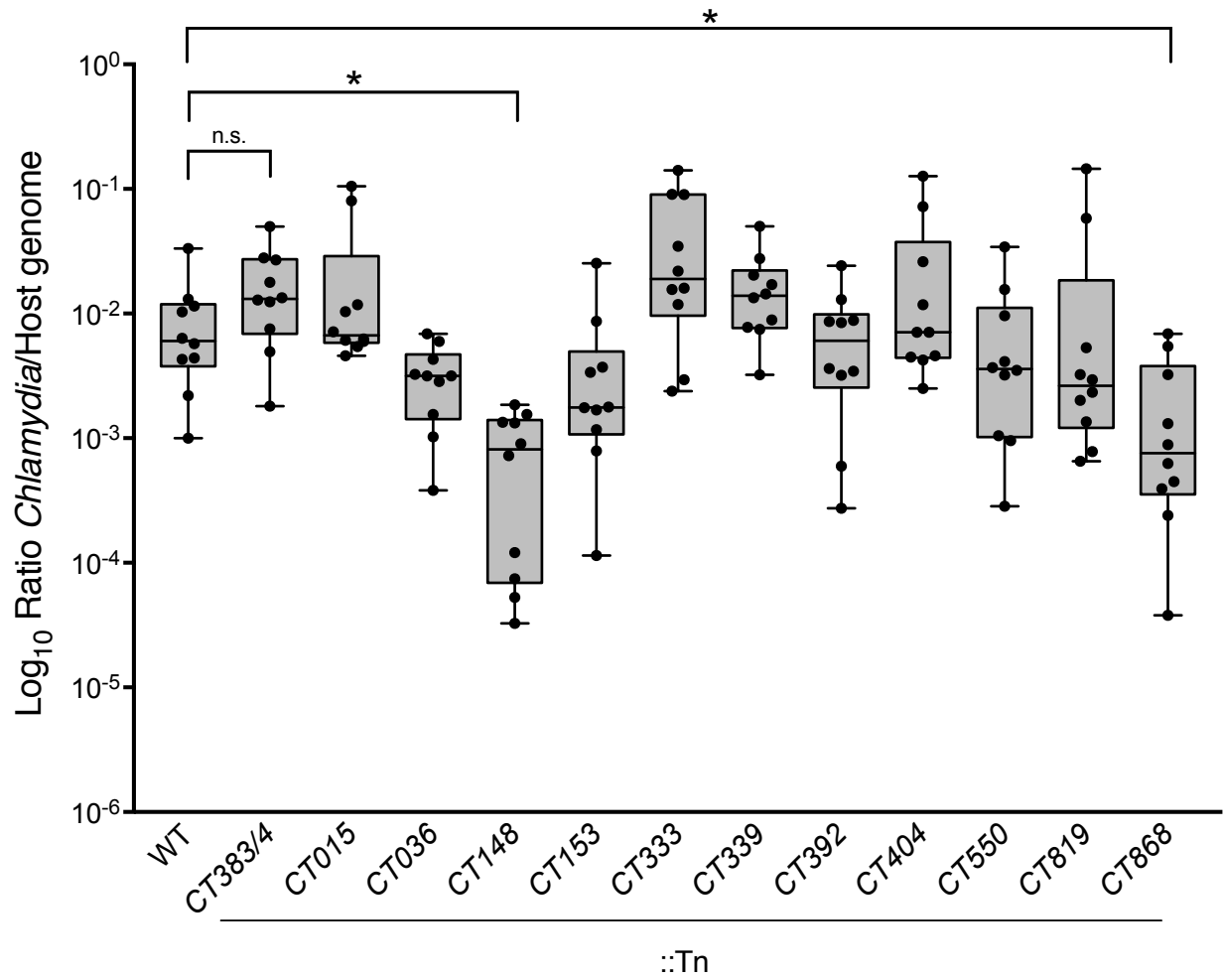


Figure 3.8. *In vivo* infections using *C. trachomatis* Tn mutants with a transcervical mouse infection model. Female C57BL/6 mice were infected transcervically with 5×10^5 IFU of either WT *C. trachomatis* or one of the Tn mutants listed above. Five days post infection, genital tracts were harvested and DNA from uterine horns purified. Droplet digital PCR was used to calculate *Chlamydia* DNA relative to host and ratios are shown as box-and-whisker with scatter plots from 10 mice. * $p < 0.05$, ** $p < 0.005$, unpaired t-test.

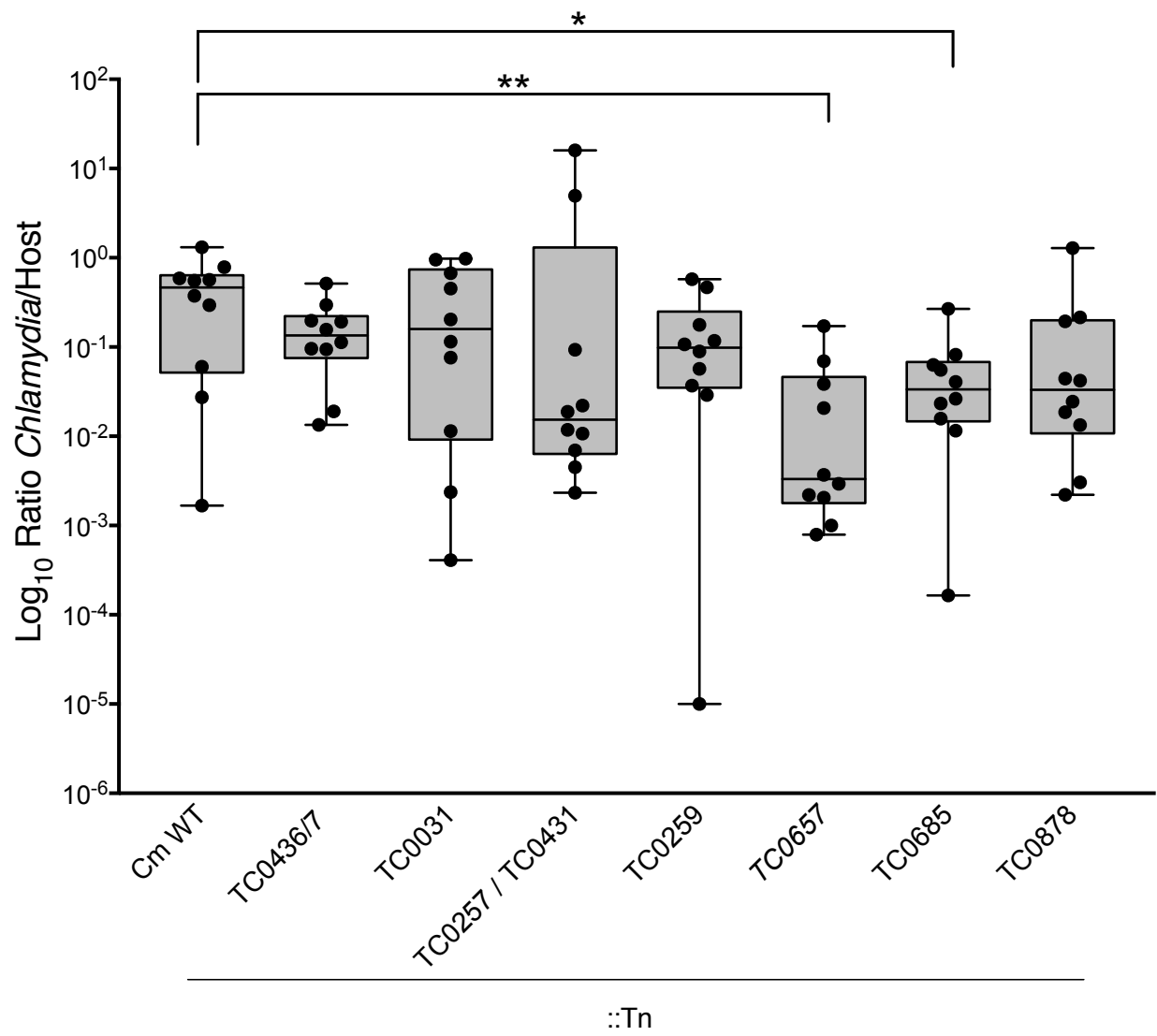


Figure 3.9. *In vivo* infections using *C. muridarum* Tn mutants with a vaginal mouse infection model. Female C57BL/6 mice were infected vaginally with 1×10^5 IFU of either WT *C. muridarum* or one of the Tn mutants listed above. Nine days post infection, genital tracts were harvested and DNA from uterine horns purified. Droplet digital PCR was used to calculate *Chlamydia* DNA relative to host and ratios are shown as box-and-whisker with scatter plots from 10 mice. * $p < 0.05$, unpaired t-test.

Chapter III.

Discussion

There have been many recent firsts in the area of genetically modifying *Chlamydia trachomatis*, including chemical mutagenesis (Kari, Goheen et al. 2011, Nguyen and Valdivia 2014, Kokes, Dunn et al. 2015), group II intron gene disruption systems (Johnson and Fisher 2013), allele replacement (Binet and Maurelli 2009, Mueller, Wolf et al. 2016), and inducible gene expression (Wickstrum, Sammons et al. 2013, Bauler and Hackstadt 2014). The development and validation of a transposon mutagenesis system using the *HimarI* transposase, as presented here, provides another first and a key advancement in the *Chlamydia* field. The data presented demonstrates that the transposase is functional within *Chlamydia* and the use of the non-replicative plasmid results in mutant *C. trachomatis* and *C. muridarum* clones containing mostly single genomic insertions. The insertion sites are readily discovered and characterized by common PCR-sequencing based techniques. Additionally, these insertion mutations are stable and can be maintained under selective pressures, limiting chances of reversion. A primary benefit of this limited insertion, random mutagenesis approach, is the ability to associate a specific genetic disruption to a resulting phenotype. This approach is among the more prominent and successful techniques for microbiological discovery. As demonstrated by the insertion disruption in the MACPF (CT153/TC0431) in both *C. trachomatis* and *C. muridarum* and subsequent biological studies related to mammalian infection in *Chlamydia* presented here, the scientific contributions of this molecular tool and approach are expected to be very useful for *Chlamydia* studies as well.

Notwithstanding these benefits, the present study also highlights the extremely low level of transformation efficiency associated with *Chlamydia* and the current limitation for large-scale transposon mutagenesis studies. In these studies, approximately five to six transposon insertion clones were obtained per transformation. This low transformation efficiency may suggest an extreme density of genes essential for growth and completion of the chlamydial developmental cycle within cell culture conditions (*in vitro*). However, recent random chemical mutagenesis study by Valdivia and colleagues (Nguyen and Valdivia 2014) reported that 84 protein-coding genes incurred a non-sense mutation with limited growth effect, albeit within the context of other mutations. This supports that a density of essential genes is likely not the limiting factor for the few transposon mutant clones generated per transformation. Moreover, four genes (*CT036/CTL0291*, *CT333/CTL0587*, *CT339/CTL0593*, and *CT392/CTL0648*) within this transposon mutant library were also disrupted in the chemical mutagenesis study, suggesting that the disruption of many more genes may be tolerated *in vitro* (*i.e.* cell culture). This also suggests that these non-essential *in vitro* genes may be more critical during the more evolutionary shaping environment of mammalian infection. Indeed, whereas *CT036::Tn*, *CT148::Tn*, *CT153::Tn* and *CT868::Tn* did not show obvious growth defects *in vitro*, all four of these mutants showed decreased ability to establish infection in the mouse transcervical model and designates a baseline for the biological processes these gene products may be essential in. Further the initiation of *in vivo* studies with the *C. muridarum* library will be of particular significance to our understanding of *Chlamydia* pathogenesis, using these Tn mutants to investigate the relative importance of particular gene products during ascension and potential differences in host-tropisms and co-evolutionary development within the mouse.

This study generated a relatively small library of thirteen transposon insertion mutants in *C. trachomatis* and 26 mutants in *C. muridarum* that support the majority of disrupted gene products are not essential for *in vitro* growth, with the exclusion of a disruption in hypothetical protein, CT696. These libraries contain a number of noteworthy disruptions that could have been expected to have a more observable effect on bacterial growth in cell culture conditions. CT868 encodes a putative deubiquitinase termed Cdu1 and the Tn inserted in the carboxyl-terminus coding region rendering a 297 a.a. truncation (401 a.a. wild-type sequence). *Chlamydia* encode two deubiquitinating proteins, Cdu1 and Cdu2, which both contain the same catalytic triad of SUMO-specific proteases and are capable of binding probes specific of deubiquitinating enzymes (Misaghi, Balsara et al. 2006). The *CT868::Tn* insertion occurs just before this active site and is expected to disrupt the deubiquitination activity of this protein. Cdu1 has been demonstrated to be capable of inhibiting host NF- κ B: a central regulator of numerous immune responses including cytokine secretion and T-cell proliferation, and a vital defense against intracellular pathogens (Misaghi, Balsara et al. 2006, Le Negrate, Krieg et al. 2008, Gerondakis, Fulford et al. 2014). The targets and biological role of Cdu2 remains unknown and may provide redundant function to explain the absence of observable fitness defects *in vitro*. This Tn insertion *CT868::Tn*, will enable future studies to evaluate the potential redundancy and protein target specificity for Cdu1 and Cdu2, as well as their effect on infectivity and pathogenesis in an animal model. Further, the *C. muridarum* Cdu2 ortholog TC0259 also incurred an insertion, albeit outside the proposed active site of the protein. This disruption appeared to have no effect on the growth or infectivity of the organism *in vitro* or *in vivo*, supporting the proposed essentiality of an intact active site for a functional gene product.

Another notable disruption that was associated with no observable growth defect was *CT819::Tn*. *CT819* encodes a protein with a domain containing high similarity to the apoptosis Bax inhibitor-1. Through Bax-1 inhibition, not only is Bax-mediated apoptosis halted, but intracellular defenses, such as reactive oxygen levels and cytosolic acidification, are abrogated (Robinson, Clements et al. 2011). Given previous observations related to apoptosis inhibition during *Chlamydia* infections (Byrne and Ojcius 2004), this insertion might be expected to result in a more noticeable effect on growth; however, microscopic analysis revealed no obvious defect in inclusion size or bacterial morphology (Figure 3.6). Protein topology analysis indicated that *CT819* is a seven-pass integral membrane protein with no signal peptide (data not shown). Furthermore, it does not appear to encode a calcium-binding motif, as most BI-1 proteins are predicted to mediate calcium flux. This may suggest that in spite of proposed homology, *CT819* may have other roles in chlamydial pathogenesis besides apoptosis inhibition. Along these lines, studies have shown that *Chlamydia* is capable of upregulating Mcl-1, an anti-apoptotic member of the Bcl-2 family, which may provide sufficient anti-apoptotic activity *in vitro* and account for the negligible deficiency of *CT819::Tn* growth in these conditions (Rajalingam, Sharma et al. 2008).

Phenotypically, the most striking was the observable growth and morphological deficiencies of *CT696::Tn* (Figure 3.7). First classified as a phenotypic slow-grower, *CT696::Tn* took approximately 8 days until visible inclusions were detected by phase microscopy and subsequent analysis by confocal microscopy identified a decreased number of RB/EBs as well as the absence of nuclear localization typically observed with *Chlamydia* inclusions. Due to genetic placement near the known type three secretion (T3S) effector protein *CT694*, it has been hypothesized that *CT696* may be part of an operon, playing a role in T3S; however, recent work

suggests this may not be the case. Using a heterologous T3S system with *Yersinia enterocolitica* (da Cunha, Milho et al. 2014, Mueller and Fields 2015), secreted levels of the CT696 hypothetical gene product were not observed and Fields *et al.* showed transcription of *CT696* was found to occur independent of *CT694*. CT696 is a *Chlamydiaceae* conserved hypothetical protein with no sequence or conserved domains shared outside of this family. Using the bacterial localization tool PSORTb (Gardy, Laird et al. 2005), CT696 was predicted as neither a membrane-bound or T3S protein but as a cytosolic protein. While more in-depth analysis is required to evaluate the role and function of CT696 on growth and inclusion formation, this observation provides support for these efforts as well as a good example of the utility of this transposon approach for the discovery of genes important to the biology of *Chlamydia*.

Finally, whereas limited *in vitro* analysis of growth and development have been completed, the few *C. muridarum* Tn mutants that have been evaluated *in vivo* showed one mutant with decreased recoverable organisms, TC0657, a glucose-6-phosphate isomerase. The carbon metabolism pathways in *Chlamydia* have perplexed researchers for years and recent studies have once again shifted the paradigm via discovery of actively transcribed metabolic enzymes, unaltered growth phenotypes when host ATP acquisition is restricted, and evidence of chlamydial metabolism present during active growth whereas host ATP/ADP exchange is dynamic during initial and persistent infections (Tipples and McClarty 1993, Iliffe-Lee and McClarty 1999, Shaw, Dooley et al. 2000, Gérard, Freise et al. 2002). As such, while there are studies undergoing to assess growth and development of the *TC0657::Tn* mutant *in vitro*, it is fathomable that a disruption of the glucose-6-phosphate isomerase would result in altered infectivity *in vivo*. These studies will thus serve to further strengthen an argument for energy

acquisition as it relates to mammalian infections, as well as promote the discovery of mechanisms used for metabolism in *Chlamydia*.

In summary, this development of a novel transposon system that is functional in *Chlamydia* is a major leap-forward and a valued addition to the growing repertoire of genetic manipulations in this field. It is expected to have profound impact on the discovery of biologic and pathogenesis related genes, enabling association of single gene insertion with evaluated phenotypes. The study has highlighted the current inefficiency in transformation in *Chlamydia*, which will need to be overcome for large-scale mutagenesis studies. Despite this shortcoming, there is much promise for this technique and its associated applications, as evident from the support for the chlamydial MACPF (*CT153/TC0431*) in establishment of mammalian infections using both the vaginal and transcervical infection models for *Chlamydia*. There are also high expectations that this system will be applicable and functional for studies in other *Chlamydia* species and serovars (e.g. *C. trachomatis* serovar D), thus enabling animal studies and analysis of more clinically relevant strains for the discovery of virulence factors and potential therapeutic targets.

Chapter IV.

Genetic Disruption of the Hypothetical Protein CT339 Reveals an Essential Role in Lateral Gene Transfer in *Chlamydia*

Abstract

Chlamydia species are the causative agent for a number of globally relevant diseases including blinding trachoma and pelvic inflammatory disease. In spite of a reduced genome, variable host and tissue-tropisms are observed between species. Sequence analysis has revealed that lateral gene transfer played a critical role in evolutionarily shaping these genomes and altering virulence between species. Despite this important biological process, the components responsible for lateral gene transfer and DNA uptake in *Chlamydia* are virtually unknown. The development of a novel transposon mutagenesis system for use in *Chlamydia* resulted in a transposon insertion in the C-terminal half of CT339. This gene encodes a protein displaying structural similarity to the DNA-uptake protein ComEC, in spite of having limited sequence similarity. This Tn disruption rendered the mutant incapable of DNA acquisition when grown in cell culture, supporting a role in DNA uptake in *Chlamydia*. These studies describe the structural homology of the hypothetical protein CT339 to ComEC and support a functional role for CT339 in DNA uptake—identifying the first protein component essential for lateral gene transfer in *Chlamydia*.

Chapter IV

Introduction

In spite of a single chromosome and relatively small genomes compared to eukaryotic organisms, bacteria exhibit extensive amounts of genetic variation; both among and within species. Whereas rapid generation times may result in higher probabilities of mutations leading to modification or inactivation of genes, these do not account for the acquisition of large genomic islands and antibiotic resistance genes observed in a number of bacteria including Gram-positives such as *Bacillus*, Gram-negatives like *Helicobacter* and *Salmonella*, and even atypical bacteria such as *Mycoplasma* (Ochman, Lawrence et al. 2000). Similarly, it has been hypothesized that without exogenous genetic procurement, irreversibly deleterious mutations will accumulate to the point a population becomes extinct, more commonly known as Muller's ratchet (Koonin, Makarova et al. 2001, Koonin 2016). The only mechanisms for avoiding this evolutionary annihilation is the ability to obtain genes with redundant functions to those deleteriously mutated either through attaining a functional copy of the same gene or new gene(s) resulting in a similar outcome, albeit potentially through a modified pathway. Further, the ability of an organism to gain new genes leading to entirely novel functions such as the ability to metabolize a new carbon source or eliminate a common antibiotic results in increased fitness and may promote selectivity and conservation of these genes throughout evolution of the organism (Koonin 2016).

Horizontal (lateral) gene transfer, the movement of genetic material by means other than parent to offspring (vertical), has been identified in nearly every bacterial species, particularly due to the abundant methods available for transfer into bacterial species. Transposons, Plasmid

uptake via conjugation, bacteriophage and molecular parasites are all classified as major components of the “mobilome”—any type of movable DNA (Siefert 2009). Through each of these mechanisms, the transfer of genes from distantly related bacteria shapes not only the genomes of these bacteria, but phenotypically alters the individual strains upon which the genetic elements have been acquired. For example, enteropathogenic and enterohemorrhagic strains of *E. coli* have diverged from commensal *E. coli* due to the acquisition of regulatory and virulence proteins via HGT (Mellies, Barron et al. 2007). Likewise, the horizontal acquisition of over 10 pathogenicity islands within *Salmonella* contribute to the virulence of the organism with many serotype-specific islands contributing to the differential infectivity observed between serotypes: the principal example of this is observed between *Salmonella* Typhimurium and *Salmonella* Typhi in which the horizontally acquired pathogenicity island 7 in *S. Typhi* promotes systemic infections, whereas the absence of this island in *S. Typhimurium* limits the pathogen to gastrointestinal disease (Nair, Alokam et al. 2004).

The mechanisms for which horizontal gene transfer via transformation are highly described for both Gram-positive and Gram-negative bacteria, with few mechanistic gaps remaining. For both, exogenous DNA is guided to the cell membrane by a pilus where it is degraded into single-stranded DNA (ssDNA) by a DNA receptor coupled with nucleases or DNA separating proteins. This ssDNA is then internalized into the cytoplasm through the transmembrane pore, ComEC, where it incorporates into the bacterial genome through homologous recombination (Johnston, Martin et al. 2014). This incorporation is often identifiable by flanking repeat sequences, changes in GC content within segments as compared to the remainder of the genome, and the presence of partial operons from distant bacterial species (Zaneveld, Nemergut et al. 2008).

Among the global pathogen *Chlamydia*, it has been observed that lateral gene transfer played a critical role in shaping the genomes and altering virulence between species. This fundamental mechanism drives diversity of the immunodominant major outer membrane protein (OmpA) as well as polymorphic membrane proteins (Pmps), antibiotic resistance genes and other loci essential for infectivity and development (Gomes, Bruno et al. 2004). Moreover, lateral gene transfer occurs readily between strains, often resulting in enhanced tissue tropism and fitness against host defenses (Demars, Weinfurter et al. 2007, DeMars and Weinfurter 2008, Jeffrey, Suchland et al. 2010, Jeffrey, Suchland et al. 2013). Despite this importance, the components that participate in the process for lateral gene transfer and DNA uptake are virtually unknown. Similarly, many aspects of *C. trachomatis* pathogenesis are poorly understood. This is largely due to the limitation of few genetic tools that have been available for *Chlamydia* and the inability to experimentally demonstrate the importance of specific gene products to biological and infectious processes.

The development of a novel transposon mutagenesis system for use in *Chlamydia* led to the ability to directly linking single genes products to phenotypic alterations through random genetic disruptions. One of these disruptions, an insertion into the C-terminal half of CT339, led to the investigation of the protein product and its functional roles in chlamydial biology. It was observed that this protein contains homology to an inner membrane DNA uptake protein, ComEC from Gram-positive bacteria and subsequent follow-up experiments confirmed the disruption led to the loss of lateral gene transfer between strains. These studies describe the first experimental evidence of a component essential for natural competence and DNA uptake exhibited by *Chlamydia* and identify a functional role for the hypothetical protein CT339.

Chapter IV

Methods and Materials

Chlamydial strains and propagation. *C. trachomatis* serovar L2 434/Bu were propagated as described above. Briefly, L929 mouse fibroblast cells (ATCC CCL-1) were grown to confluency using RPMI medium 1640 supplemented with 5% Heat-inactivated FBS plus 10µg/mL gentamycin (Fisher Scientific, Pittsburg PA) and infected with purified EBs of either WT or Tn mutant CT339::Tn (generation method described in chapter 3). Cultures were allowed to grow for up to 48 hours at 37°C, 5% CO₂. Percent infectivity was assayed by IFA microscopy and EBs were harvested as previously described (Mukhopadhyay, Clark et al. 2004) using a series of centrifugation and sonication steps to disrupt the host-cells and release EBs. Once isolated, EBs were either frozen immediately or further purified using Omnipaque™ (Barrington, IL) and ultracentrifugation. All EBs were stored in sucrose phosphate glutamate (SPG) media at -80°C. Clonal isolates were obtained as previously described (Suchland, Bourillon et al. 2005).

Bioinformatic analysis of CT339. BLAST analysis and subsequent query within the Conserved Domain Database (Marchler-Bauer, Derbyshire et al. 2015) resulted in hits within the competence superfamily and multidomain hits for ComEC. Multiple sequence alignments were generated using ClustalW and 10 variable species hits within the Clusters of Orthologous Groups of proteins (COG0658) (Tatusov, Galperin et al. 2000). The following proteins were used; NCBI accession numbers shown in parenthesis: competence protein ComEC family protein *Shigella dysenteriae* 1617 (YP008850592.1), competence locus E *Helicobacter pylori* Hp P-26 (EJC51989.1); competence protein ComEC *Enterococcus faecium* DO (YP006375852.1); ComE

Synechocystis sp. PCC 6803 (BAA17126.1); comEC *Listeria monocytogenes* EGD-e (CAC99560.1); ComE operon protein 3 *Bacillus subtilis* subsp. *subtilis* str. 168 (NP 390435.1); hypothetical protein AGR C 2573 *Agrobacterium tumefaciens* str. C58 (NP354400.1); competence protein *Neisseria meningitidis* MC58 (NP273744.1); hypothetical protein CT339 *Chlamydia trachomatis* D/UW-3/CX (NP 219846.1); ComEC/Rec2 family protein *Clostridium botulinum* A str. ATCC 3502 (YP 001255462.1); ComEA *Neisseria meningitidis* (CAB44958); ComEA protein *Helicobacter bilis* (WP_004084273); competence ComEA *Shigella dysenteriae* 1617 (EFP70107); competence protein ComGA *Enterococcus faecium* (WP_002304768); competence protein ComGA *Listeria monocytogenes* (WP_009933475); ComGA *Bacillus subtilis* (BAA12533); Multispecies competence protein ComGB (WP_002286180); Multispecies: competence protein ComGB Bacilli (WP_048681721); competence protein ComGB *Listeria monocytogenes* (WP_010990101). Multispecies: type IV pilus secretin PilQ *Neisseria* (WP_016686778); Type 4 pili secretin pilQ (outer membrane porin) *Shigella dysenteriae* 1617 (AHA67697); Multispecies: pilus assembly protein Pile *Neisseria* (WP_002214937); ComGC *Bacillus subtilis* (BAA12535). Five different hydropathy prediction programs were used to predict topology for CT339: HMMTOP v2.0 (Tusnady and Simon 2001); MEMSAT-SVM (Buchan, Ward et al. 2010); TMHMM2.0 (Krogh, Larsson et al. 2001); TOPPRED (Claros and von Heijne 1994); SPLIT (Juretic, Zoranic et al. 2002). Transmembrane illustrations were generated and modified using Protter v1.0 (Omasits, Ahrens et al. 2013).

Generation of recombinant clones for assessment of bacterial competence. All experiments with tetracycline-resistant *Chlamydia* described in this work were reviewed and approved by the National Institutes of Health Recombinant DNA Advisory Committee (University of

Washington). Recombination experiments were performed as previously described (Suchland, Sandoz et al. 2009). Briefly, sets of individual shell vials were seeded with McCoy cells and subsequently co-infected with combinations of drug-resistant strains: *C. trachomatis* L1/tet^R (NCBI accession number ACUI01000000), *C. suis* R19/tet^R, *C. trachomatis* serovar F/tet^R, and either *C. trachomatis* IGR::Tn bla^R or *C. trachomatis* CT339::Tn bla^R. Cultures were incubated for 40 hours post-infection in the absence of antibiotics then detached using -80°C/37°C freeze-thaw. Recombinants were isolated by infecting 96 new shell vial monolayers with 1 mL freeze-thaw lysates and treating with both penicillin and tetracycline and passaged until dually resistant clones were detected. Recombinant clones were propagated and cloned by limiting dilution and subsequently evaluated for identification of serovar-specific OmpA. DNA from each clone was harvested and subject to WGS. Recombinant clones were propagated and purified by limiting dilution.

Whole Genome sequencing. DNA was extracted from purified EBs using the Qiagen Blood and Tissue Kit. Manufacturer's instructions were used with minor modifications. In brief, purified EBs were aliquoted with Buffer ATL and boiled for 10 minutes at 96°C. Proteinase K was added and the reactions were incubated at 56°C for 1 hour. After incubation, Buffer AL and ethanol (96-100%) was added and mixed thoroughly by vortexing. The reactions were added onto a provided DNeasy Mini spin column and collection tube, and centrifuged at 6000 x g. Two wash steps were then performed using Buffer AW1 and AW2 each with centrifugation. Finally, reactions were incubated for with Buffer AE for 20mins at room temperature, centrifuged and eluate was saved. For quality control, each sample was verified using a spectrophotometer (Denovix Ds-11 FX+) to quantify DNA. Extracted DNA was prepared for sequencing at the

Genome Sequencing Core at the University of Kansas where library preparation and further quality control was completed. Samples were multiplexed and run on the Illumina Miseq PE100. Paired end reads were generated with a Phred score ($>Q30$) of 95.44%. Reads were then demultiplexed and analyzed on the Geneious software suite. Total read coverage was calculated as a function of the proportion of reads that mapped to the WT reference genome (the total of chlamydial reads) over the total number of reads generated for the sample, including host and other contaminant DNA. A threshold of 10 reads was created to check for the depth of coverage at each base pair (2013).

Assembly and Analysis of the Recombinant Progeny. Each recombinant genome was generated using reference-guided assembly to the OmpA parental genome. Genomes were then aligned to both parental genomes and scanned for regions of homology using the Geneious global alignment tool with free end gaps and a cost matrix of 65% similarity. Initial sequence assembly was verified through reference-guided assembly to the minority parent. Regions of homology were annotated and margins were estimated based on similarity to the individual parents.

Identification of parental backbone and recombination sites. DNA from each clone was harvested and subject to PCR using primers for arbitrary regions representative of the whole genome (Table 4.1). Amplicons were sequenced using ACGT (Wheeling, IL) for identification of recombination sites.

Table 4.1: Primers used to determine parental backbone of R19/L2 recombinants

L2xR19 Recombination PCR (5'-3')		
<u>Gene</u>	<u>Primer Sequence and Designation</u>	<u>PCR Product Size (bp)</u>
<i>C. trachomatis ompA</i>	GAGCTAAACTTGCTTGCCATTC CCTGCTGAACCAAGCCTTAT	751
<i>C. suis</i> R19 <i>ompA</i>	CTAGCACAGGGAATACCACATC AAGCTTGCTCGAGACCATTTA	619
<i>C. trachomatis oppF</i>	TGCCTCCAGATGCTCAAGACTC TTGCCAAATCATCTGCACCGC	330
<i>C. suis</i> MD56 <i>oppF</i>	ACATCATGGGAGGACTCTCTAA GCGAAAGTAAATGACGGAATAGC	597
<i>C. trachomatis glgB</i>	TGATGGAGTACCTGGAGTAAGA GAGACAGCATCCACACGAATA	825
<i>C. suis</i> MD56 <i>glgB</i>	CTAGGAGAGCCTTTCGCATTTA TTGATTTAGCCTCGGTCTTCTC	763
<i>C. trachomatis CTL0600</i>	TGGAGACCATTGCTTAGGTTTAG CTTGTTCCGCAGCTTGTTTG	473
<i>C. suis</i> MD56 <i>CTL0600</i>	ACGATTCTAGGCTGTTCAAGTC GATGGGCGTGCTCTTCTAAA	592
<i>C. trachomatis CTL0592</i>	TCTAAACGCACGTTATACGAAGG TTTCCTTATCGTTATTTGGCTCAAG	200
<i>C. suis</i> MD56 <i>CTL0592</i>	CACACCGTACTGAGCATAAA ATGGTGTACAGTTATAAAGGCT	275
<i>C. trachomatis CTL0594</i>	CGCAGCATTGGAAGGATAGA GTGAGGATGTCCGAGGAAATA	381
<i>C. suis</i> MD56 <i>CTL0594</i>	CACAAGCTCACTGCCAAATC CTGTGGTATTCTCGGAGCATAA	332
<i>C. trachomatis CTL0808</i>	TGTAGGAGCGAAATCGAAAGG GGAAGTCACGAAGGAGCTATG	560
<i>C. suis</i> MD56 <i>CTL0808</i>	CTGAGGCGGCAATAGAACT ACACTGCTTCTTGACATACTC	441
<i>C. trachomatis CTL0542</i>	AGTTTCGAGTTGGATCCGGTAGTTC AGCAATTCCCTCTCCGATACCTG	149
<i>C. suis</i> MD56 <i>CTL0542</i>	TCCGGATGTTGTGCAAGAA CTACGGGTATTGGAGAAGGAATC	247

Chapter IV

Results

In silico analyses of CT339 support functional prediction as DNA uptake protein ComEC.

Given the importance of lateral gene transfer in the evolution and adaptation of *Chlamydia* and the paucity of identifiable gene candidates that may play a role in this fundamental process, the Tn insertion in CT339 was of particular interest. CT339 shares limited sequence similarity to any single protein outside of *Chlamydia*; however, it contains similarity to multiple competence-associated protein families and conserved domains, including ComEC (E-values ranging from 2.33e-03 to 4.40e-24). In both Gram-negative and positive bacteria, ComEC plays a key role in transporting ssDNA into the cytosol of the bacterial cell during natural DNA acquisition (Berge, Moscoso et al. 2002, Draskovic and Dubnau 2005). To further investigate the possibility of CT339 encoding a ComEC homolog, *in silico* analyses were performed.

Using the Conserved Domain Database, 10 randomly selected proteins from COG658 with an E-value less than 6E-3 were used to demonstrate the diversity of organisms containing the competence motif identified within ComEC, including CT339 from *C. trachomatis*. These integral membrane proteins typically have 9-12 transmembrane helices and a conserved metal-binding motif HΦxxΦSGΦH (Φ indicate hydrophobic residues, Figure 4.1).

Topology modeling of CT339 using five hydropathy programs resulted in a range of 8 (SPLIT) to 11 (MEMSAT) predicted transmembrane segments, similar to the proposed 9-12 transmembrane regions of ComEC from *Bacillus* (Draskovic and Dubnau 2005). As previously described for ComEC, HMMTOP prediction identified a large N-terminal loop within CT339 spanning residues 94-256 with a potentially stabilizing disulfide bond formed by C148-C167 as

ComE operon protein 3 *Bacillus subtilis* subsp. *subtilis* str. 168
 ComEC *Listeria monocytogenes* EGD-e
 Competence protein ComEC *Enterococcus faecium* DO
 ComEC/Rec2 family protein *Clostridium botulinum* A str. ATCC 3502
 ComE *Synechocystis* sp. PCC 6803
 Competence locus E *Helicobacter pylori* Hp P-26
 Hypothetical protein AGR C 2573 *Agrobacterium tumefaciens* str. C58
 Competence protein ComEC family protein *Shigella dysenteriae* 1617
 Competence protein *Neisseria meningitidis* MC58
 Hypothetical protein CT339 *Chlamydia trachomatis* D/UW-3/CX

G	V	V	H	L	A	I	S	G	L	H
G	V	V	H	L	A	I	S	G	L	H
G	L	L	H	L	S	L	S	G	L	H
G	V	I	H	A	S	V	S	G	F	H
G	L	S	H	F	A	A	S	G	Y	Q
G	I	N	H	L	A	I	S	G	F	H
G	L	A	H	I	A	I	S	G	L	N
G	T	A	H	L	A	I	S	G	L	H
G	L	T	H	L	S	I	S	G	L	H
G	L	S	H	L	S	V	S	G	W	H

Figure 4.1. *CT339* shows sequence similarity competence homology to ComEC. Multiple sequence alignment of 10 competence proteins identified as containing the multi-domain ComEC from the Conserved Domain Database. COG0658 was used to identify 10 bacterial species with similar competence domains and an E-value $<6e-3$. Hashtags indicate conserved histidine metal-binding motifs. Conserved hydrophobic residues are highlighted in cyan.

well as the competence domain containing the conserved metal-binding motif HΦxxΦSGΦH (Figure 4.2A and B). For all five hydropathy programs, an extracellular N-terminal domain and intracellular C-terminal domain were predicted, similar to that of ComEC (Draskovic and Dubnau 2005). Contrary to the predicted topology of ComEC, none of the predictive transmembrane helices for CT339 display an amphipathic character nor are any segments predicted to be buried parallel to the membrane surface (Figure 4.2A). Based on this *in silico* data, a transmembrane topology model was designed (Figure 4.2B) supporting the structural homology between CT339 and ComEC, specifically within conserved competence domains and the N-terminal loop. Further experimental analysis suggests CT339 may be functioning similar to ComEC as a component of bacterial competence and may lead to identification of a mechanism for DNA uptake among similar obligate intracellular pathogens.

Requirement of CT339 for DNA uptake via lateral gene transfer.

Chlamydia has been demonstrated to be naturally competent, acquiring and integrating DNA within or between certain *Chlamydia* species (Suchland, Sandoz et al. 2009). Importantly, the mechanisms and components for this process are poorly understood. The transposon insertion in CT339 provided an opportunity to experimentally evaluate the importance of this gene product in DNA uptake and support the prediction of CT339 as a functional homolog to ComEC. Lateral gene transfer during co-infections, including the transfer of specific antibiotic resistance genes, has been demonstrated to occur between *C. trachomatis* strains (intra-species) as well as between *C. suis* and *C. trachomatis* (inter-species) (Suchland, Sandoz et al. 2009). To evaluate the importance of CT339 in lateral gene transfer, intra-species co-infections followed by dual antibiotic selection were performed to detect the transfer of antibiotic markers between parental

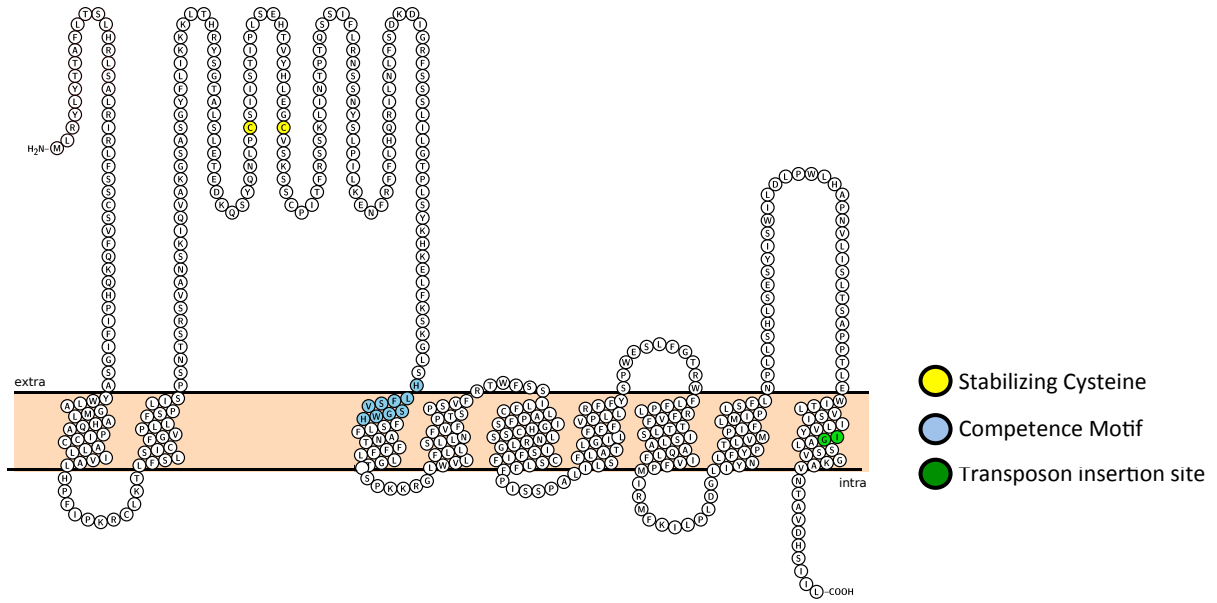
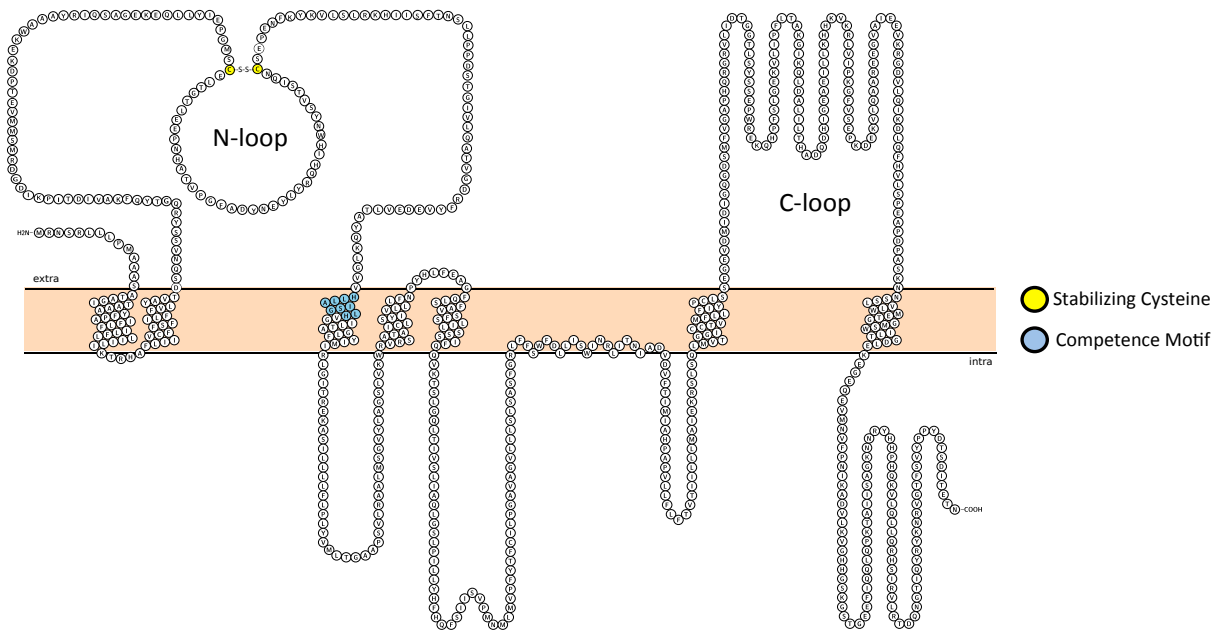


Figure 4.2 CT339 shows structural homology to ComEC. A and B) Hydropathy plots of both ComEC from *Bacillus subtilis* (A) and CT339 from *Chlamydia* (B). Both predicted competence proteins display a large extracellular N-terminal loop stabilized by two cysteines (highlighted in yellow) and an extended C-terminal loop. The competence domain as identified by the conserved domain database is highlighted in blue.

strains. Recombination rates and acquisition of antibiotic resistance markers were assessed by parental OmpA serotype (e.g. L1 vs. L2).

Intra-species co-infections and dual antibiotic selection with tetracycline resistant *C. trachomatis* L1 (L1/*tet*^R) and either CT339::*Tnbla*^R (339) or the transposon control mutant CT383/4::*Tnbla*^R (IGR) were performed. When *C. trachomatis* L1/*tet*^R and IGR were co-infected, roughly equal amounts of recombinant progeny displaying either parental OmpA (L1 or L2) were observed. In contrast, when the same L1/*tet*^R parent was co-infected with 339, nearly 100-fold lower progeny displaying the L2 OmpA were observed (Table 4.2). While this supported the importance of CT339 (*comEC*) in DNA acquisition, the low levels (~10³ IFU of L2) might suggest that this gene product is not essential for the process.

An alternate hypothesis was posed to explain the low level of L2 OmpA positive samples: that a region including the L2 *ompA* gene, in addition to the CT339::*Tnbla*^R marker, was transferred and integrated into L1 parent organisms containing intact, functional CT339. To investigate this hypothesis, whole genome sequencing on several recombinants from each co-infection (L1/*tet*^R vs L2 CT339::*Tnbla*^R or L2 IGR::*Tnbla*^R) were performed. As indicated in Fig. 4.3, L1/*tet*^R and L2 IGR::*Tnbla*^R crosses yielded genomes that predominantly reflect the OmpA serotype. Specifically, L2 or L1 OmpA positive samples had an average of 84% L2 or 78% L1 genomic composition, respectfully. In contrast, L2 OmpA positive samples generated after a L1/*tet*^R x L2 CT339::*Tnbla*^R co-infection had relatively minimal L2 genome in the resulting clones, with an average genomic L2 composition of 36% (Fig. 4.3), less than half as much as observed in IGR v L1 crosses. Matching the levels of antibiotic resistance transfer in CT339 intact samples (IGR), L1 positive OmpA samples had an average of 80% L1 genomic

Table 4.2. OmpA Phenotype of L1/L2 Tn Recombinant Progeny

L1/<i>tet</i>^R x IGR::<i>Tn bla</i>^R			
Cross	L1 OmpA IFU	L2 OmpA IFU	% L2 Progeny
1	9 x 10 ³	7 x 10 ³	43.8
2	8 x 10 ³	6 x 10 ³	42.9
3	9 x 10 ⁵	6 x 10 ⁵	40
L1/<i>tet</i>^R x CT339::<i>Tn bla</i>^R			
1	5 x 10 ³	7 x 10 ³	1.4
2	6 x 10 ³	8 x 10 ³	1.3
3	9 x 10 ³	6 x 10 ³	0.66

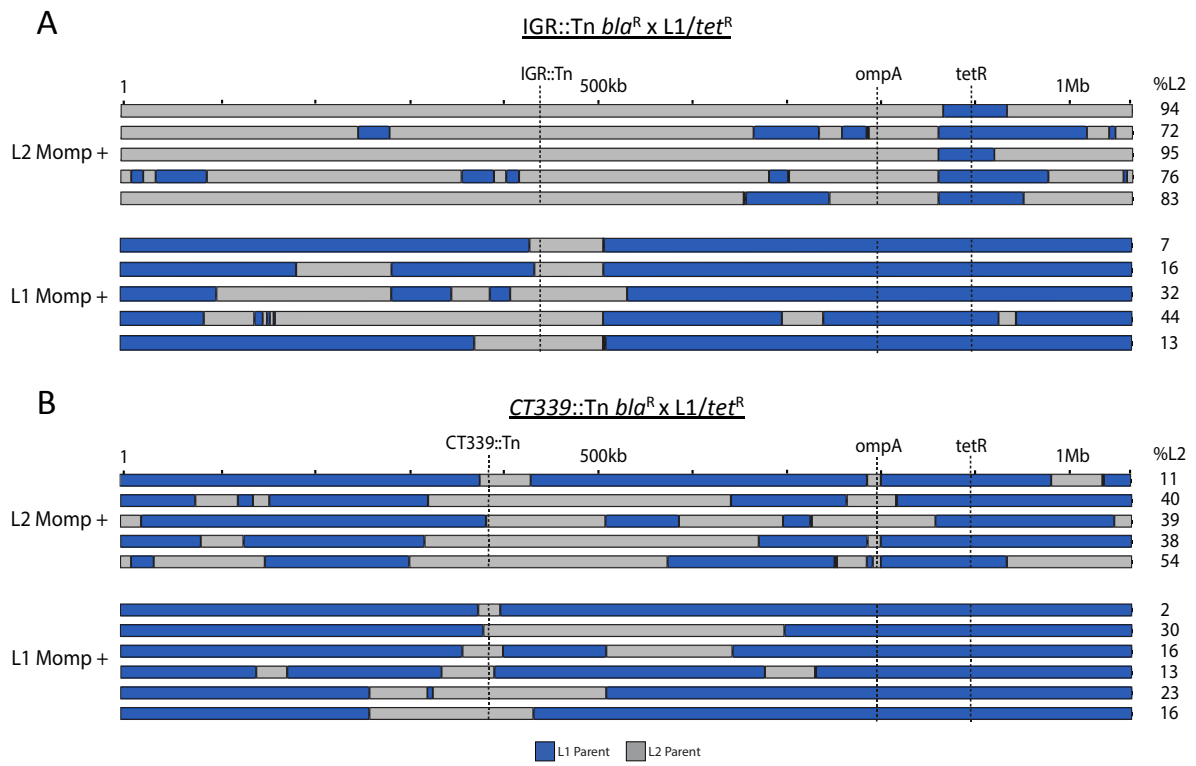


Figure 4.3. Assessment of lateral gene transfer using Tn mutants. Schematic representation of recombinant progeny genomes. Blue bars represent the regions of the genome from the L1/*tet*^R parent. Gray bars represent regions from either L2 *CT383/4::Tn bla*^R (IGR) or L2 *CT339::Tn bla*^R parent. A) Progeny genomes from crosses between tetracycline resistant *C. trachomatis* L1 and *CT383/4::Tn bla*^R (IGR). B) Progeny genomes from crosses between tetracycline resistant *C. trachomatis* L1 and *CT339::Tn bla*^R (339).

composition (Fig. 4.3). These data support the hypothesis that DNA regions containing L2 OmpA and the *CT339::Tnbla^R* marker, was most likely acquired by L1 parent clones.

The generation of L1/*tet^R*/L2 *CT339::Tnbla^R* clones provided an excellent opportunity to further investigate the possibility of incomplete *CT339* requirement for DNA acquisition and genome incorporation. These clones were used in co-infections with the parent L2 *CT339::Tnbla^R* clone with dual antibiotic selection. If *CT339* is essential for DNA acquisition and incorporation, then all resulting clones should be only L1 OmpA positive. If *CT339* is only partially required, then it would be expected to observe mixed L1 and L2 OmpA populations (i.e. the *tetR* marker transferred and incorporated into the L2 genome or L2 OmpA transferred and incorporated into the L1 genome). Three independent co-infections revealed that only L1 OmpA positive inclusion was observed with an extensive amount of IFUs ($\sim 5 \times 10^7$) per evaluated cross (data not shown). Additionally, transformation of the L2 *CT339::Tnbla^R* was also attempted using an inducible GFP plasmid (pTLR2-GFP), and chloramphenicol for selection. Every attempt to transform L2 samples that had an intact chromosomal *CT339* were successful using the inducible plasmid. In contrast, none of the attempts to transform L2 *CT339::Tnbla^R* with the plasmid were successful (data not shown). Taken together, these data support the hypothesis that *CT339* is critical for DNA uptake and is likely serving as a ComEC functional homolog in *Chlamydia trachomatis*.

To further address the functionality of *CT339* in lateral gene transfer, similar co-infections and dual selection experiments were performed with tetracycline resistant *C. trachomatis* serovar F (*F/tet^R*). Using a more distant species relative reduces the challenge of delineating highly similar genomic crossover regions, although this also reduces recombination rates. Three independent crosses and selections were performed with *C. trachomatis* *F/tet^R* and

either *C. trachomatis* L2 IGR::Tn *blaR* or L2 CT339::Tn *blaR*. As observed in the L1 x L2 IGR experiments (Table 4.2), approximately half of the inclusions exhibited either OmpA F or L2 seroreactivity (Table 4.3). Similar to the L1/*tet*^R vs L2 CT339::Tn crosses, more than 99.94% of the inclusions were positive for only OmpA F (Table 4.3), strongly supporting the reliance of CT339 for DNA uptake in *C. trachomatis*.

Finally, inter-species co-infections with *C. suis* R19*tet*^R and *C. trachomatis* CT339::Tn*bla*^R or CT383/4 (IGR)::Tn*bla*^R were performed. After an initial growth without antibiotics, lysates from co-infections were diluted among 96 individual vials in the presence of dual antibiotic selection. Infections were passaged until visible inclusions were observed and subsequently evaluated for identification of either *C. suis* or *C. trachomatis*-specific OmpA. The genetic variation of the two *Chlamydia* species allowed for accurate identification of source genome regions that were recombined following lateral gene transfer.

For co-infections with *C. suis* R19*tet*^R and *C. trachomatis* IGR::Tn*bla*^R, an average of approximately 15 dual resistant cultures were detected (range 8-23). Of these, the vast majority (94% with an average of 14 resistant cultures; range 8-21) of the resulting cultures were positive for *C. trachomatis* OmpA, supporting that lateral transfer of the *tet* gene from *C. suis* into *C. trachomatis* genome occurs readily in the control IGR::Tn*bla*^R clone (Figure 4.4A). In contrast, very few (6% with an average of 1 resistant culture; range 0-2) of these co-infections yielded dual resistant cultures that were positive for *C. suis* OmpA (Figure 4.4A). This suggests that the recombination regions surrounding the IGR::Tn*bla*^R may be poorly compatible with the *C. suis* genome. When co-infections with *C. suis* R19*tet*^R and *C. trachomatis* CT339::Tn*bla*^R were performed, dual resistant clones that stained positive for *C. suis* OmpA were more detected (average 14.4 cultures with a range of 4-45) supporting the ability of the *bla* resistance to the *C.*

Table 4.3. OmpA Phenotype of F/L2 Tn Recombinant Progeny

F/tet^R x IGR::<i>Tn bla</i>^R			
Cross	F OmpA IFU	L2 OmpA IFU	% L2 Progeny
1	5 x 10 ³	4 x 10 ³	44.4
2	5 x 10 ³	3 x 10 ³	37.5
3	3 x 10 ³	3 x 10 ³	50
F/tet^R x CT339::<i>Tn bla</i>^R			
1	1 x 10 ⁴	4 x 10 ⁰	0.04
2	2 x 10 ⁴	7 x 10 ⁰	0.04
3	9 x 10 ³	5 x 10 ⁰	0.06

suis R19*tet*^R genome at this site (*CT339::Tn*) is more readily accepted. Strikingly, no lysates from any of these five co-infection experiments stained positive for *C. trachomatis* OmpA. This observation supports the hypothesis that disruption of *CT339* renders the *C. trachomatis* recipient cell incapable of DNA uptake during lateral gene transfer.

To provide a more comprehensive analysis of the recipient genome's species, sequence analysis of three loci (*glgB*, *oppF*, and *Q499_0583/CTL0808/CT546*) distributed around the genome was performed (Figure 4.4B). For all of the resulting progeny following co-infection with *C. suis* and *C. trachomatis*, all amplicon sequences matched the species defined by the OmpA seroreactivity. While this approach is a relatively crude genome investigation, it does support that antibiotic resistant loci (*tet*^R or *bla*^R) from a given donor species is transferred into a predominately species-specific genome. While it is highly unlikely that spontaneous mutations would occur that rendered *Chlamydia* cultures resistant to β -lactams or tetracycline, PCR sequence analysis of antibiotic resistant genes (*bla* and *tet*) of all dual-resistant lysates supported their presence and contribution to resistance. To further determine the directionality of antibiotic resistance transfer between strains, whole genome sequencing will be performed. Additional experiments utilizing recombination with the L1 *C. trachomatis* serovar are also underway, as previous studies have shown *C. trachomatis* serovars readily exchange genetic material, whereas recombination between species (*C. trachomatis* and *C. suis*) has resulted in exceedingly low recombination rates (Jeffrey, Suchland et al. 2013).

A

Species Serotypes (OmpA) of Parental and Recombinant Progeny

Parental Crosses	Recombinant Progeny	Percent Total Progeny ¹
<i>C. trach</i> IGR::Tnbla ^R X <i>C. suis</i> R19tet ^R	<i>C. trach</i> IGR::Tnbla ^R , tet ^R	94.1 ± 3.6
	R19tet ^R , bla ^R	5.9 ± 3.6
<i>C. trach</i> CT339::Tnbla ^R X <i>C. suis</i> R19tet ^R	<i>C. trach</i> CT339::Tnbla ^R , tet ^R	ND
	R19tet ^R , bla ^R	100

¹ Average percentage for five individual crosses

² N.D. – None detected

B

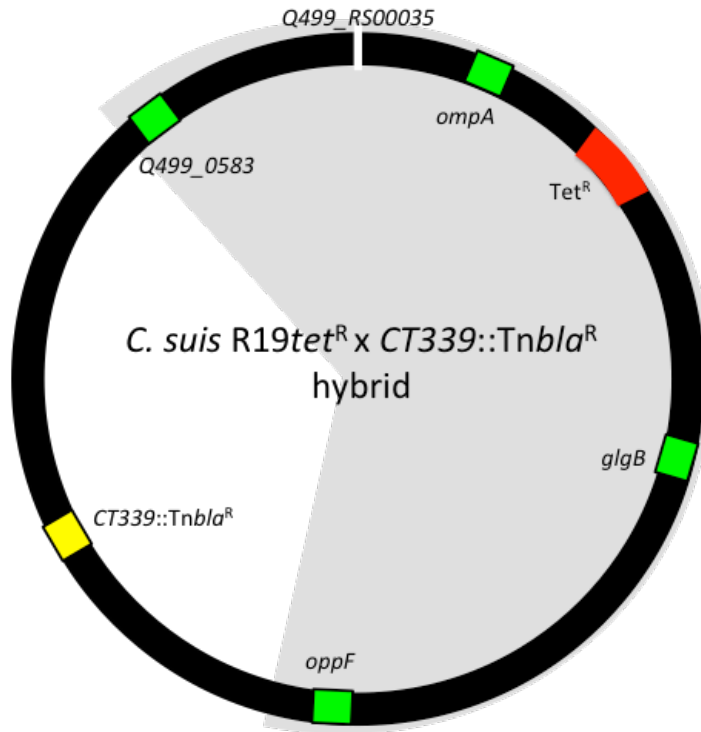


Figure 4.4. Recombination with CT339::Tn results in lack of DNA uptake. A) Serotypes of parental strains and progeny from 5 independent recombination crosses. Isolates are displayed as the percent wells showing growth with respective progeny OmpA stain. B) Graphical representation *C. suis* R19 x CT339::Tn hybrid progeny. Arbitrary genes throughout the genome were amplified and sequenced for identification of direction of genetic transfer. Red region indicates Tet resistance specific to *C. suis*. and *C. suis* Q499_RS00035 indicated at the top of the representative genomes for reference. Green boxes indicate genes sequencing for *C. suis* MD56 reference strain. Yellow box indicates hypothesized Tn recombination region including CT339::Tn^{bla}^R. Gray shadow indicates region of *C. suis* backbone based on arbitrary gene sequencing.

Chapter IV

Discussion

For nearly all bacteria, the ability to uptake exogenous DNA is essential for genetic variability resulting in beneficial evolutions in fitness. Horizontal gene transfer both between and within species has resulted in bacteria obtaining new means for survival as well as pathogenicity. In the Gram-positive bacteria *Staphylococcus aureus* and *Clostridium difficile*, both toxins and antibiotic resistance genes appear to have been horizontally acquired, resulting in two of the most prevalent hospital-acquired, multi-drug resistant human pathogens (Juhas 2015). Along with gaining genes through plasmid and transposon insertion, a new mechanism for genetic transfer known as “microbial superhighways” has been identified in which bacteria are shown to form nanotubules for transfer of proteins, small molecules and nucleic acids (Dubey and Ben-Yehuda 2011, Schertzer and Whiteley 2011). These tubules have been shown to occur in eukaryotes, both plants and animals, and given the hypothesized ancestral symbiosis between *Chlamydomonas* and plant species, it may not be farfetched to believe these tubules may have been responsible for early horizontal gene transfer between them (Bordenstein and Reznikoff 2005).

Within *Chlamydia*, there is no evidence of conjugation machinery, only six bacteriophages have been identified with none known to infect the globally prevalent *C. trachomatis* (Pawlikowska-Warych, Sliwa-Dominiak et al. 2015), and while there is evolutionary evidence for transposon insertion, this is only present in *C. suis* strains containing a tetracycline resistance cassette (Marti, Kim et al. 2017). Thusly, the only explanation for genetic diversity among species, including the presence of plant-derived transport proteins, variability among

Pmps, and acquisition of eukaryote-only helicases, is the ability for *Chlamydia* to readily take up foreign DNA from the environment. Indeed, both within the laboratory and within nature, *Chlamydia* has been shown to be capable of transformation, although at relatively low levels compared to other bacteria (Gomes, Bruno et al. 2004, Demars, Weinfurter et al. 2007, Suchland, Sandoz et al. 2009).

What has remained intangible is how *Chlamydia* procures foreign DNA. To date, the machinery associated with DNA uptake: pilins, porins, nucleases etc., have not been functionally annotated within *C. trachomatis*. Through random mutagenesis using transposons, however, we have identified a key component in DNA uptake: the ComEC homolog CT339.

We have shown *in silico* that CT339 structurally resembles ComEC as described in *Bacillus* (Draskovic and Dubnau 2005) with multiple transmembrane domains, the competence motif and a large N-terminal loop containing potentially stabilizing cysteines (Figure 4.2). Co-infection experiments using the transposon insertion mutant in CT339 (CT339::Tn) showed an observable absence of lateral gene transfer capability, providing compelling support for the essential contribution of this gene product to this fundamental evolutionary process. According to the protein family database (pfam) architecture, genes encoding proteins containing this competence motif are typically encoded near a lactamase-superfamily protein that are predicted to regulate the competence operon (Charpentier, Faucher et al. 2008). Genome analysis of *C. trachomatis* identified ribonuclease Z (CT346), a lactamase-superfamily protein, neighboring CT339 and suggesting that this protein may be involved in the chlamydial competence system. As previously mentioned, other potential components involved in DNA uptake in *Chlamydia* are less evident. Pili or pseudopili predominantly facilitate binding and transport of DNA to the bacterial surface, although no proteins sharing any sequence similarity to pili homologs were

identified in *Chlamydia*. Interestingly, *Helicobacter pylori* is unique among naturally transformable bacteria in that it does not use pili but instead relies upon a dedicated type IV secretion system for DNA uptake (Hofreuter, Odenbreit et al. 2001). Gram-negative bacteria typically transport dsDNA through an outer membrane secretin channel (e.g. PilQ), which is then processed into ssDNA with periplasmic protein, ComEA. Single stranded DNA is then transported through inner membrane protein ComEC and into the bacterial cytoplasm where it is bound by RecA or DprA (Johnston, Martin et al. 2014). Direct pairwise BLAST analysis of *C. trachomatis* with numerous gram-negative secretin homologs (PilQ) revealed sequence similarity (E-values $<10^{-9}$) to only a type II and type III secretion outer membrane proteins, GspD (CT572) and YscC (CT674), respectively. Similarly, no protein with similarity to various ComEA or DprA homologs was identified through sequence based pair-wise analysis.

Clearly there is much to be learned about the *Chlamydia* DNA uptake system although it is safe to state that, similar to *H. pylori*, *Chlamydia* is likely an exception to the general DNA uptake model. Current follow-up studies to further functionally annotate CT339 are ongoing, including attempting to transform *CT339::Tn*, identifying biophysical properties of CT339 such as extracellular loops and stabilizing cysteine, as well as more in-depth recombination assays. Nonetheless, this study marks not only the first relevant application of transposon mutagenesis to assign functionality to a given gene product but also identifies a central component for the process of DNA uptake shown to occur in *Chlamydia*, providing a foundation for explicating the remaining machinery used amongst this process.

Chapter V.

Discussion

Over the past 20 years, reported *Chlamydia* cases have been steadily increasing, yet few advances in prophylactic treatment or antibiotic therapy have become available. This impediment is largely due to the scarcity of understanding in terms of basic biology of the organism. With a poor comprehension of the functional and biological roles gene products may be playing in survival of the bacterium, construction of appropriate pathogen-specific treatments is encumbered. Moreover, the obligate intracellular niche in which *Chlamydia* survive has inhibited the ability of basic genetic studies in order to identify these essential components. While there are currently no threats of antibiotic resistance in the human-adapted *C. trachomatis* species, evidence of resistant gene acquisition and chronic, persistent infections insinuate forthcoming public health complications which, if not anticipated, have the potential for devastating outcomes (Suchland, Sandoz et al. 2009, Schoborg 2011). The research presented herein takes a foundational approach to biology, developing the tools necessary for understanding these genetic correlates of infection. Two different approaches were developed and assessed to encompass the gain- and loss-of-function approaches to understanding the roles of both individual genes, and integral loci in chlamydial infectivity. One gene product identified using these techniques, CT339, was also the subject of successive analysis for functionality in the competence pathway of DNA uptake, demonstrating the feasibility of the approach and resulting discoveries that can be made upon its utilization.

Advantages and disadvantages of homologous recombination as a means for identifying genetic correlates.

With a highly reduced genome, it is predicted that chlamydial species have retained only those genes essential for growth and pathogenesis. In spite of evolutionary host-speciation of the bacteria, remarkably high levels of genetic similarity are retained. Between *C. trachomatis* and the mouse-adapted species *C. muridarum*, over 98% genome similarity is present, yet upon infection of mice, each shows dramatically different phenotypes with regard to pathogenesis (Thomson, Holden et al. 2008, Stephens, Myers et al. 2009). What remain unclear are the specific genetic variances between these species that account for this disparity.

The process by which *Chlamydia* ascend into the UGT has been the topic of debate for decades with a general agreement that it is likely multifactorial, involving both the bacterial induction of inflammation, fluid movement based on host reproductive cycles, and interactions between pathogens and commensal microenvironments (Pal, Hui et al. 1998, Gupta, Wali et al. 2014). Along the same lines, it is expected that numerous genes and regulatory components are involved in *Chlamydia* establishing mammalian infections. Bearing in mind the high similarity in genetic content between *C. trachomatis* and *C. muridarum*, the 2% of dissimilarity in genome syntony may account for these contrarities, found within a ~50kb region of the genomes adequately named the plasticity zone (Read, Brunham et al. 2000). The immense size of this variable region, along with the challenges encountered using historic genetic manipulation techniques led to the development of an innovative approach to genome assessment using *Chlamydia*'s natural ability to undergo homologous recombination (Demars, Weinfurter et al. 2007, Suchland, Sandoz et al. 2009). The major advantage for using recombination is the ability

to transfer large genomic clusters using less complicated genetic modification techniques: organisms capable of recombination need only selective markers for isolating progeny; the remaining tools required are encoded in the bacteria itself. Additionally, while reverse genetic approaches are useful in identifying roles of single genes through loss-of-function (Griffiths, Miller et al. 2000), these methods are less than ideal for the investigation of proposed multi-genetic physiognomies, such as the mechanisms behind chlamydial ascension. Independent of gene products contributing to ascension, it is anticipated that non-coding regulatory components are also having an effect, as has been observed in other intracellular pathogens including *Brucella* and *Coxiella* (Ahmed, Zheng et al. 2016). Targeted genetic disruption would not detect these features and, as such, indispensable genetic constituents may be overlooked.

The methods by which recombinant clones were generated efficaciously resulted in isolation of triple-antibiotic resistant progeny. Two of these resistance markers were of *C. trachomatis* origin, while ofloxacin resistance was conferred from *C. muridarum* (Figure 2.2). Unfortunately, resistance to fluoroquinolones is easily generated through a point mutation in the DNA gyrase subunit A (Barnard and Maxwell 2001) and previous work has shown keeping chlamydial growth under antibiotic pressures for as little as four passages resulted in increased levels of antibiotic resistance (Sandoz and Rockey 2010). This was the only marker for *C. muridarum* among recombinant progeny, thus it was not surprising to find that for nearly half the recombinant library, ofloxacin resistance was not the result of *C. muridarum* gene integration but instead spontaneous mutations in *C. trachomatis gyrA*. Brief analyses and whole genome sequencing of one clone confirmed that genomes from spontaneous-mutation ofloxacin-resistant isolates were entirely of *C. trachomatis* origin, showing no recombination with *C. muridarum*. Reflecting on how few passages are necessary to generate this spontaneous mutation, it is

expected that multi-pass cultures or samples that underwent extensive clonal dilutions would be mainly *C. trachomatis*. In fact, it was calculated that between the two most recent recombination experiments, of ~18 vials kept under selection and serially passaged, 12 isolates sequenced positively for *C. trachomatis gyrA* (data not shown). Future studies in which a less mutable target or more than one *C. muridarum* identifiable marker are incorporated will assist in the generation of recombinant clones. Selection of isolates based on *C. muridarum* reactive IncA, for example, would provide a more stable identifier and, as it flanks the right terminus of the plasticity zone, may provide a novel locus for assessment of ascension.

Looking beyond technical insufficiencies that can easily be overcome, more profound disadvantages to using homologous recombination require attention. Whole-genome analysis has shown *Chlamydia* has a non-uniform genome, meaning that genes associated with similar functions or biological roles may not be in close proximity to each other (Thomson, Holden et al. 2008). As it is hypothesized that ascension presumptively requires numerous genes and regulatory components, it may be that these are not located within the same loci. Supposing this is the case, observable gain-of-function phenotypes could be very difficult to obtain. Similarly, the regions of *C. muridarum* that may integrate into *C. trachomatis* or vice versa are relatively unrestricted in size; fragments of *C. muridarum* can span anywhere between IncA and *rpoB* (Figure 2.2), a nearly 200 kb region. Assuming a gain-of-function mutant is isolated from a hybrid spanning this region, the follow-up analysis to determine which precise components are responsible for the phenotype is unreasonable. Rather, a second round of recombination utilizing this isolate, in which hybrids are generated with more narrow regions of integration, would be more feasible. This all results in tedious experimental set ups and maintenance of cocultures, while remaining conscious of the spontaneous mutations that may arise with continued

propagation. Instead, the generation of parental strains encoding a diverse array of resistance markers would be more useful.

The analysis of the hybrid clones that were generated through the crosses did reveal a feature common among the genomes. Practically all isolates encoded *trpA* from *C. trachomatis* and *TC0440* from *C. muridarum*. As mentioned before, it is perhaps due to the span of homologous sequences present within this region, however, it could be due to a potential toxicity associated with cytotoxin integration into a *C. trachomatis* genome. Numerous conversations between chlamydiologists have expounded on the difficulties of cloning recombinant cytotoxins and disruptions of the cytotoxins have not been done in which all three are activated (Newman 2015). Prior to this work, attempts to recombinatorally modify the cytotoxins have not been attempted. It may be that these genes encode redundant functions, therefore phenotypes will not be observed with single and double knockouts. Although, seeing as the chlamydial genomes are highly reduced, it is likely species would not maintain genes with redundant functions unless absolutely necessary and, if absolutely necessary that these gene products be maintained, disruptions of one or two should result in distinguishable phenotypes. Therefore, it is improbable that the cytotoxins are functioning in the same roles. What remains clear is that downstream recombination from *TC0440* does not result in ascension detectable seven days post infection (Figure 2.7). The variation in organisms observed in cervix tissues may be indicative of bacterial clearance and therefore analysis taken at earlier timepoints are necessary. Previous studies using transcervical administration of *C. trachomatis* shows peak of infection between 3 and 7 days post-infection (Gondek, Olive et al. 2012, Fischer, Harrison et al. 2017) therefore future analysis in which organs are harvested at roughly 5 dpi should be implemented.

In order to identify the numerous genetic correlates associated with *Chlamydial* ascension, homologous recombination offers advantages over all other molecular genetic approaches: the ability for integration of large genomic regions and relatively simple methods obtaining hybrids. These genetic regions have the potential to incorporate both genes and regulatory sequences essential for novel functions. While considerations should be made regarding the technical aspects of obtaining recombinants, the generation of more diverse selection markers placed uniformly throughout the genome will provide more opportunities for targeted recombination incorporating specific loci of interest.

Transformation efficiency is a limiting factor for library generation.

Transposon mutagenesis has been paramount in terms of generating mutant libraries and identifying genes essential for growth and survival in a number of organisms. These include: *Neisseria* (Mendum, Newcombe et al. 2011), *Streptococcus* (Le Breton, Belew et al. 2015), and *Mycobacterium* (Griffin, Gawronski et al. 2011, Long, DeJesus et al. 2015), with up to 30% of genes screened determined to be essential for bacterial fitness. For *Chlamydia*, this is the first report of a functional Tn mutagenesis system and therefore the initial generation of libraries for the assessment of gene essentiality. It is expected that a saturation point will be achieved, in which recurrent insertions within repeated genes will be observed, and all genes capable of sustaining mutations will have been identified. To expedite the process, using a derivation of the formula for Poisson's Law (Zilsel, Ma et al. 1992), the number of Tn mutations required to disrupt every gene within the genome can be theorized. The formula for which is as follows:

$$N = \frac{\ln(1 - \emptyset)}{\ln(1 - f)}$$

in which \emptyset is the probability of hitting a particular gene of interest, f is the average size of each gene within the genome and N is the number of mutant isolates required to hit said probability (\emptyset). *Chlamydia* contains a ~1Mb genome with ~900 coding sequences, resulting in an average gene size of ~1000 bp (Stephens, Kalman et al. 1998, Nunes, Borrego et al. 2013), giving an f value of 1/1000 or 0.001. Naturally, not every gene will tolerate an insertion; disruption of those absolutely essential for growth and survival such as DNA replication, translation, and other critical cellular processes would result in an inability for *Chlamydia* to survive in cell culture and thus, no growth would be observed. Previous work from Bastidas et al. showed *C. trachomatis* L2 is capable of withstanding at least 99 nonsense mutations within 84 open reading frames (Bastidas and Valdivia 2016). In order to attain similar numbers of mutants, using the aforementioned formula, nearly 105 successful transformations must be accomplished e.g. 10% of the genome = $\frac{\ln(1-0.1)}{\ln(1-0.001)}$.

As described in chapter 3, one transformation split into 12-wells resulted in between 4-6 wells showing successful transformants and growth of Tn mutants. It would therefore take between 17-26 transformation experiments (i.e. 12-well plates) to meet the same 10% genome mutation reported by Bastidas (Bastidas and Valdivia 2016). Given that disruptions in essential genes will result in lack of bacterial growth entirely, attempting to mutate *every* gene in the genome, such that $\emptyset = 0.99$, will provide the theoretical opportunity to hit every gene and identify all genes capable of sustaining an insertion. To achieve this, however, nearly 5000 successful transformation experiments are required, or over 1000, 12-well plates (99% of the genome = $\frac{\ln(1-0.99)}{\ln(1-0.001)}$).

Given the sheer workload associated with these protocols— 4 rounds of serial passages onto new monolayers every 36 hours— the numbers of concurrently running experiments

becomes an additional limitation for expansion of the library. The typical workflow for mutant isolation takes ~1 week and if, for instance, the maximum number of 12-well plates that can be maintained at any given time is 5, over 200 weeks' worth of labor is required. While this may vary between individuals, it is clear the remarkably low transformation efficiency is the bottleneck for library generation and a 'brute-force' approach for obtaining mutants is less than ideal.

To overcome this challenge, a number of approaches may be implemented. The addition of a fluorescent marker such as GFP within the *C. muridarum* library allows for potential sorting of transformed bacteria via FACS. Early separation of Tn mutants allows for less serial passaging and elimination of competition between potential "slow growers" and those mutants that grow similar to WT—allowing for isolation of more dramatically altered phenotypes that may have been outcompeted and lost during standard practices. Similarly, using an inducible plasmid to turn expression of the transposase on and off may allow for generation of higher numbers of mutants. One undesirable outcome of this approach, however, may be the presence of multiple insertions; precise control of transposase expression and/or removal of the plasmid containing the transposase then becomes crucial. Re-introducing *only* the transposase on an inducible plasmid to an aliquot of an already isolated Tn mutant may also overcome this challenge. Within the *C. trachomatis* library described in chapter 3, it has been confirmed only one copy of the *bla^R* gene is present. With only one copy, unrestrained transposase expression would simply result in the "jumping" of *bla^R* from one location into another rather than the presence of multiple insertions. In using this method, multiple unique, single insertions may be isolated, simply by controlling the expression of the transposase. Regardless of method

implemented, it is imperative for library generation and subsequent studies that the transformation efficiency be improved dramatically.

Transposon mutant *CT696* is highly specific to chlamydial biology.

As previously mentioned, the development of a functional *HimarI* transposon system provided the field with the ability to generate libraries of random, single-gene disruption mutants for associated phenotype-genotype correlations. Libraries in both *C. trachomatis* and *C. muridarum* not only expanded the repertoire of available mutants for functional analyses; it also provided the ability to identify differential roles genes may be playing in species-specific phenotypes. For example, both libraries contain insertions within the MAC/Perforin from the PZ, which, as described extensively above, marks the region of highest genetic diversity. Differential functions or expression profiles based on speciation could provide valuable information on roles neighboring genes may have in chlamydial biology. Wherein this research describes the progressive, broad applications this system offers, it is not without its shortcomings. Transformation efficiency remains a bottleneck for expanding the library and current protocols enrich for disruptions in genes nonessential for growth in cell cultures. Disruptions that show dispensability for *in vivo* infections as well provide little contribution in understanding chlamydial pathogenesis. Instead, emphasis should be placed on poorly-characterized gene products such as the disruption observed in *CT696::Tn*. With no sequence homology to proteins outside *Chlamydia* and no discernable conserved domains or motifs, it is clear this protein is specific to this pathogen and may identify a novel therapeutic target or biological process. Unfortunately, the severe defect in development complicates subsequent studies in that stocks of

culture are difficult to obtain. Nonetheless, it is clear that inclusion morphology is severely impaired in this mutant, suggesting CT696 may be playing a role in modifying the endocytic vacuole or maintaining the inclusion upon entry. Four different protein predictors were unable to identify worthwhile features within CT696, including ProSite, InterPro or Pfam and seven different topology predictors failed to identify signal peptides or transmembrane regions (TOPCONS, OCTOPUS, Philius, PolyPhobius, SCAMPI, SPOCTOPUS and PDB-homology). Similarly, three different effector protein prediction programs found no evidence that CT696 may be a secreted effector protein, highlighting the specificity it must have to an obscure aspect in chlamydial biology.

Transposon mutants overcome deficiencies observed in recombination techniques.

One unexpected benefit the transposon libraries provided was the ability to overcome aforementioned deficiencies in homologous recombination. Currently, the primary disadvantage to recombination is the lack of selectable markers throughout the chlamydial genomes. Ofloxacin, tetracycline, and rifampicin resistance were the only isolates available for use. As shown in Figures 3.3 and 3.4, sites of Tn insertion occur all across the genome, providing new regions for directed recombination. Current work generating Tn/recombination mutants is underway to assess whether more localized pressures using β -lactamase can drive integration of all three cytotoxins into a *C. trachomatis* backbone, without the concern of spontaneous generation of antibiotic resistance. Analogously, isolation of *C. muridarum* hybrids in which all three cytotoxins have been lost may answer the posing question of redundancy, toxicity, and essentiality for survival and infectivity.

Identifying how CT339 enables DNA uptake.

Recently, strong experimental evidence supports that CT339 is functioning similar to ComEC from *Bacillus* but how this protein fulfills this function remains poorly understood. Computational analysis on *Bacillus* ComEC proposes that this protein forms a homodimer and both disulfide bonds and the intramembrane competence motif maintain this structure (Draskovic and Dubnau 2005). The prediction of an OB superfamily fold, a structural component responsible for DNA binding, and a C-terminal β -lactamase super family domain in ComEC both provide *in silico* explanations for how this protein functions in DNA uptake. In telomere end-binding proteins, OB folds dimerize to allow for binding of ssDNA (Theobald and Schultz 2003). Two independent studies, nearly ten years apart, conclude that ComEC likely dimerizes in a manner similar to these end-binding proteins, in that homology modeling predicted the presence of similar OB domains in the N-terminal loop (Baker, Simkovic et al. 2016). This N-terminal loop was previously predicted to exist extracellularly, which is consistent with a proposed role in DNA binding. While HHpred and covariance-assisted modeling was not done on CT339 like ComEC (Baker, Simkovic et al. 2016), the presence of a large extracellular N-terminal loop supports that this, too, is functioning in a manner similar to telomere end-binding proteins (Figure 4.2B). If ComEC and CT339 dimerize as predicted, the pore formed by the competence motif would result in both N- and C-terminal extracellular loops functioning together for DNA uptake. The Tn insertion, then, would disrupt proper folding and subsequent loop interactions, inhibiting DNA binding and dramatically reducing the ability for DNA uptake, as observed (Draskovic and Dubnau 2005). Future experiments assessing the stabilizing nature of the N-terminal cysteines, the potential of pore-formation by the competence motif, and any

interaction of extracellular loops with ssDNA would provide conclusive experimental evidence for CT339 as a homolog to ComEC.

The predicted β -lactamase super family domain within ComEC was similarly identified using Pfam and homology modeling predicted a catalytically active zinc-binding pocket within this domain, functioning as a nuclease (Baker, Simkovic et al. 2016). As was previously described for DNA uptake, the endonucleases such as EndA must first cleave double stranded DNA into ssDNA (Johnston, Martin et al. 2014). In *Bacillus*, the endonuclease responsible for this cleavage has yet to be identified; the presence of a nucleating domain on ComEC thus accounts for this absent enzyme (Baker, Simkovic et al. 2016). Unfortunately, both Pfam and SMART analysis of CT339 did not identify any similar nucleating domains (Schultz, Milpetz et al. 1998). Considering the presence of a downstream lactamase-superfamily protein CT346, it is not surprising that this domain may be absent from CT339. It could be that gene duplication or fragmentation resulted in CT346 becoming independent of CT339 and the lack of uniformity across the genome corroborates the ~10 genes between these. CT345, for example, is a hypothetical protein within this region showing domain conservation to the ATP-dependent DNA ligase, CDC9 superfamily of proteins (accession cl25417). This superfamily has shown to play important roles in recombination during DNA joining (Wilkinson, Day et al. 2001). Presently, no data existing here support this speculation. However, these observations express the feasibility of applying Tn mutagenesis for the discovery of complete, functional complexes; such as the entire competence apparatus. Overall, using Tn mutagenesis led to the discovery of CT339, pinpointing exploration of the genome to this locus for identification of the remaining constituents involved in DNA uptake. Exploration and identification of the remaining

competence-associated components will provide more thorough understandings of the mechanism behind this vital evolutionary process.

References

- (2013). MiSeq Sample Sheet Quick Reference Guide. I. Illumina. San Diego. **15028392 Rev. J.**
- Abdelrahman, Y. M. and R. J. Belland (2005). "The chlamydial developmental cycle." FEMS Microbiol Rev **29**(5): 949-959.
- Ahmed, W., K. Zheng and Z. F. Liu (2016). "Small Non-Coding RNAs: New Insights in Modulation of Host Immune Response by Intracellular Bacterial Pathogens." Front Immunol **7**: 431.
- Alarcon, I., D. J. Evans and S. M. J. Fleiszig (2009). "The Role of Twitching Motility in Pseudomonas aeruginosa Exit from and Translocation of Corneal Epithelial Cells." Investigative ophthalmology & visual science **50**(5): 2237-2244.
- Ammar, I., Z. Izsvak and Z. Ivics (2012). "The Sleeping Beauty transposon toolbox." Methods Mol Biol **859**: 229-240.
- Bagel, S., V. Hüllen, B. Wiedemann and P. Heisig (1999). "Impact of gyrA and parC Mutations on Quinolone Resistance, Doubling Time, and Supercoiling Degree of Escherichia coli." Antimicrobial Agents and Chemotherapy **43**(4): 868-875.
- Baker, J. A., F. Simkovic, H. M. C. Taylor and D. J. Rigden (2016). "Potential DNA binding and nuclease functions of ComEC domains characterized in silico." Proteins: Structure, Function, and Bioinformatics **84**(10): 1431-1442.
- Barnard, F. M. and A. Maxwell (2001). "Interaction between DNA Gyrase and Quinolones: Effects of Alanine Mutations at GyrA Subunit Residues Ser(83) and Asp(87)." Antimicrobial Agents and Chemotherapy **45**(7): 1994-2000.
- Bastidas, R. J. and R. H. Valdivia (2016). "Emancipating Chlamydia: Advances in the Genetic Manipulation of a Recalcitrant Intracellular Pathogen." Microbiol Mol Biol Rev **80**(2): 411-427.

Batteiger, B. E., W. Tu, S. Ofner, B. Van Der Pol, D. R. Stothard, D. P. Orr, B. P. Katz and J. D. Fortenberry (2010). "Repeated Chlamydia trachomatis genital infections in adolescent women." J Infect Dis **201**(1): 42-51.

Bauler, L. D. and T. Hackstadt (2014). "Expression and targeting of secreted proteins from Chlamydia trachomatis." J Bacteriol **196**(7): 1325-1334.

Bavoil, P. M., R. Hsia and D. M. Ojcius (2000). "Closing in on Chlamydia and its intracellular bag of tricks." Microbiology **146 (Pt 11)**: 2723-2731.

Beare, P. A., D. Howe, D. C. Cockrell, A. Omsland, B. Hansen and R. A. Heinzen (2009). "Characterization of a Coxiella burnetii ftsZ mutant generated by Himar1 transposon mutagenesis." J Bacteriol **191**(5): 1369-1381.

Beare, P. A., K. M. Sandoz, A. Omsland, D. D. Rockey and R. A. Heinzen (2011). "Advances in genetic manipulation of obligate intracellular bacterial pathogens." Front Microbiol **2**: 97.

Beatty, W. L., R. P. Morrison and G. I. Byrne (1994). "Persistent chlamydiae: from cell culture to a paradigm for chlamydial pathogenesis." Microbiol Rev **58**(4): 686-699.

Becker, E. and J. H. Hegemann (2014). "All subtypes of the Pmp adhesin family are implicated in chlamydial virulence and show species-specific function." Microbiologyopen **3**(4): 544-556.

Benjamin, H. W. and N. Kleckner (1992). "Excision Of Tn10 From The Donor Site During Transposition Occurs By Flush Double-Strand Cleavages At The Transposon Termini." Proceedings of the National Academy of Sciences of the United States of America **89**(10): 4648-4652.

Berge, M., M. Moscoso, M. Prudhomme, B. Martin and J. P. Claverys (2002). "Uptake of transforming DNA in Gram-positive bacteria: a view from Streptococcus pneumoniae." Mol Microbiol **45**(2): 411-421.

Bernkopf, H., P. Mashiah and Y. Becker (1962). "Correlation between morphological and biochemical changes and the appearance of infectivity in FL cell cultures infected with trachoma agent." Ann N Y Acad Sci **98**: 62-81.

Bertram, R. and W. Hillen (2008). "The application of Tet repressor in prokaryotic gene regulation and expression." Microb Biotechnol **1**(1): 2-16.

Betts, H. J., K. Wolf and K. A. Fields (2009). "Effector protein modulation of host cells: examples in the Chlamydia spp. arsenal." Curr Opin Microbiol **12**.

Betts, H. J., K. Wolf and K. A. Fields (2009). "Effector protein modulation of host cells: examples in the Chlamydia spp. arsenal." Curr Opin Microbiol **12**(1): 81-87.

Binet, R. and A. T. Maurelli (2009). "Transformation and isolation of allelic exchange mutants of Chlamydia psittaci using recombinant DNA introduced by electroporation." Proc Natl Acad Sci U S A **106**(1): 292-297.

Blyth, W. A. and J. Taverne (1972). "Some consequences of the multiple infection of cell cultures by TRIC organisms." J Hyg (Lond) **70**(1): 33-37.

Bond, S. R. and C. C. Naus (2012). "RF-Cloning.org: An online tool for the design of restriction-free cloning projects." Nucleic Acids Res.

Bonner, C. A., G. I. Byrne and R. A. Jensen (2014). "Chlamydia exploit the mammalian tryptophan-depletion defense strategy as a counter-defensive cue to trigger a survival state of persistence." Front Cell Infect Microbiol **4**: 17.

Bordenstein, S. R. and W. S. Reznikoff (2005). "Mobile DNA in obligate intracellular bacteria." Nat Rev Microbiol **3**(9): 688-699.

Buchan, D. W., S. M. Ward, A. E. Lobley, T. C. Nugent, K. Bryson and D. T. Jones (2010). "Protein annotation and modelling servers at University College London." Nucleic Acids Res **38**(Web Server issue): W563-568.

Byrne, G. I. and D. M. Ojcius (2004). "Chlamydia and apoptosis: life and death decisions of an intracellular pathogen." Nat Rev Microbiol **2**(10): 802-808.

Caetano-Anolles, G. (1993). "Amplifying DNA with arbitrary oligonucleotide primers." PCR Methods Appl **3**(2): 85-94.

Camacho, L. R., D. Ensergueix, E. Perez, B. Gicquel and C. Guilhot (1999). "Identification of a virulence gene cluster of Mycobacterium tuberculosis by signature-tagged transposon mutagenesis." Molecular Microbiology **34**(2): 257-267.

Carlson, J. H., S. F. Porcella, G. McClarty and H. D. Caldwell (2005). "Comparative Genomic Analysis of Chlamydia trachomatis Oculotropic and Genitotropic Strains." Infection and Immunity **73**(10): 6407-6418.

Chakraborty, S., H. Ji, J. Chen, C. A. Gersbach and K. W. Leong (2014). "Vector modifications to eliminate transposase expression following piggyBac-mediated transgenesis." Sci Rep **4**: 7403.

Charpentier, X., S. P. Faucher, S. Kalachikov and H. A. Shuman (2008). "Loss of RNase R induces competence development in Legionella pneumophila." J Bacteriol **190**(24): 8126-8136.

Chen, I. and D. Dubnau (2004). "DNA uptake during bacterial transformation." Nat Rev Microbiol **2**(3): 241-249.

Cheng, C., A. D. Nair, V. V. Indukuri, S. Gong, R. F. Felsheim, D. Jaworski, U. G. Munderloh and R. R. Ganta (2013). "Targeted and random mutagenesis of Ehrlichia chaffeensis for the identification of genes required for in vivo infection." PLoS Pathog **9**(2): e1003171.

Cilloniz, C., S. Ewig, E. Polverino, M. A. Marcos, C. Esquinas, A. Gabarrus, J. Mensa and A. Torres (2011). "Microbial aetiology of community-acquired pneumonia and its relation to severity." Thorax **66**(4): 340-346.

Clark, T. R., A. M. Lackey, B. Kleba, L. O. Driskell, E. I. Lutter, C. Martens, D. O. Wood and T. Hackstadt (2011). "Transformation frequency of a mariner-based transposon in *Rickettsia rickettsii*." J Bacteriol **193**(18): 4993-4995.

Claros, M. G. and G. von Heijne (1994). "TopPred II: an improved software for membrane protein structure predictions." Comput Appl Biosci **10**(6): 685-686.

Cochrane, M., C. W. Armitage, C. P. O'Meara and K. W. Beagley (2010). "Towards a *Chlamydia trachomatis* vaccine: how close are we?" Future Microbiol **5**(12): 1833-1856.

da Cunha, M., C. Milho, F. Almeida, S. V. Pais, V. Borges, R. Maurício, M. J. Borrego, J. P. Gomes and L. J. Mota (2014). "Identification of type III secretion substrates of *Chlamydia trachomatis* using *Yersinia enterocolitica* as a heterologous system." BMC Microbiology **14**(1): 1-14.

Dal Conte, I., M. Mistrangelo, C. Cariti, M. Chiriotto, A. Lucchini, M. Vigna, M. Morino and G. Di Perri (2014). "Lymphogranuloma venereum: an old, forgotten re-emerging systemic disease." Panminerva Med **56**(1): 73-83.

Dautry-Varsat, A., A. Subtil and T. Hackstadt (2005). "Recent insights into the mechanisms of *Chlamydia* entry." Cellular Microbiology **7**(12): 1714-1722.

De Clercq, E., I. Kalmar and D. Vanrompay (2013). "Animal Models for Studying Female Genital Tract Infection with *Chlamydia trachomatis*." Infection and Immunity **81**(9): 3060-3067.

Deininger, P. L. and M. A. Batzer (2002). "Mammalian retroelements." Genome Res **12**(10): 1455-1465.

DeMars, R. and J. Weinfurter (2008). "Interstrain gene transfer in *Chlamydia trachomatis* in vitro: mechanism and significance." J Bacteriol **190**(5): 1605-1614.

Demars, R., J. Weinfurter, E. Guex, J. Lin and Y. Potucek (2007). "Lateral gene transfer in vitro in the intracellular pathogen *Chlamydia trachomatis*." J Bacteriol **189**(3): 991-1003.

Di Matteo, M., J. Matrai, E. Belay, T. Firdissa, T. Vandendriessche and M. K. Chuah (2012). "PiggyBac toolbox." Methods Mol Biol **859**: 241-254.

Draskovic, I. and D. Dubnau (2005). "Biogenesis of a putative channel protein, ComEC, required for DNA uptake: membrane topology, oligomerization and formation of disulphide bonds." Mol Microbiol **55**(3): 881-896.

Dreses-Werringloer, U., I. Padubrin, B. Jurgens-Saathoff, A. P. Hudson, H. Zeidler and L. Kohler (2000). "Persistence of *Chlamydia trachomatis* is induced by ciprofloxacin and ofloxacin in vitro." Antimicrob Agents Chemother **44**(12): 3288-3297.

Driskell, L. O., X. J. Yu, L. Zhang, Y. Liu, V. L. Popov, D. H. Walker, A. M. Tucker and D. O. Wood (2009). "Directed mutagenesis of the *Rickettsia prowazekii* pld gene encoding phospholipase D." Infect Immun **77**(8): 3244-3248.

Dubey, G. P. and S. Ben-Yehuda (2011). "Intercellular nanotubes mediate bacterial communication." Cell **144**(4): 590-600.

Dubnau, D. (1999). "DNA uptake in bacteria." Annu Rev Microbiol **53**: 217-244.

Dugan, J., D. D. Rockey, L. Jones and A. A. Andersen (2004). "Tetracycline resistance in *Chlamydia suis* mediated by genomic islands inserted into the chlamydial inv-like gene." Antimicrob Agents Chemother **48**(10): 3989-3995.

Fan, T., H. Lu, H. Hu, L. Shi, G. A. McClarty, D. M. Nance, A. H. Greenberg and G. Zhong (1998). "Inhibition of Apoptosis in *Chlamydia*-infected Cells: Blockade of Mitochondrial

Cytochrome c Release and Caspase Activation." The Journal of Experimental Medicine **187**(4): 487-496.

Fields, K. A. and T. Hackstadt (2002). "The chlamydial inclusion: escape from the endocytic pathway." Annu Rev Cell Dev Biol **18**: 221-245.

Finnegan, D. J. (1992). "Transposable elements." Curr Opin Genet Dev **2**(6): 861-867.

Fischer, A., K. S. Harrison, Y. Ramirez, D. Auer, S. R. Chowdhury and B. K. Prusty (2017). "Chlamydia trachomatis-containing vacuole serves as deubiquitination platform to stabilize Mcl-1 and to interfere with host defense." **6**.

Furuta, Y., K. Yahara, M. Hatakeyama and I. Kobayashi (2011). "Evolution of cagA oncogene of Helicobacter pylori through recombination." PLoS One **6**(8): e23499.

Gardy, J. L., M. R. Laird, F. Chen, S. Rey, C. J. Walsh, M. Ester and F. S. Brinkman (2005). "PSORTb v.2.0: expanded prediction of bacterial protein subcellular localization and insights gained from comparative proteome analysis." Bioinformatics **21**(5): 617-623.

Gebert, D. and D. Rosenkranz (2015). "RNA-based regulation of transposon expression." Wiley Interdisciplinary Reviews: RNA **6**(6): 687-708.

Geisler, W. M., A. Uniyal, J. Y. Lee, S. Y. Lensing, S. Johnson, R. C. Perry, C. M. Kadrnka and P. R. Kerndt (2015). "Azithromycin versus Doxycycline for Urogenital Chlamydia trachomatis Infection." N Engl J Med **373**(26): 2512-2521.

Gérard, H. C., J. Freise, Z. Wang, G. Roberts, D. Rudy, B. Krauß-Opatz, L. Köhler, H. Zeidler, H. Ralph Schumacher, J. A. Whittum-Hudson and A. P. Hudson (2002). "Chlamydia trachomatis genes whose products are related to energy metabolism are expressed differentially in active vs. persistent infection." Microbes and Infection **4**(1): 13-22.

Gerondakis, S., T. S. Fulford, N. L. Messina and R. J. Grumont (2014). "NF-[kappa]B control of T cell development." Nat Immunol **15**(1): 15-25.

Golden, M. R., W. L. H. Whittington, H. H. Handsfield, J. P. Hughes, W. E. Stamm, M. Hogben, A. Clark, C. Malinski, J. R. L. Helmers, K. K. Thomas and K. K. Holmes (2005). "Effect of Expedited Treatment of Sex Partners on Recurrent or Persistent Gonorrhea or Chlamydial Infection." New England Journal of Medicine **352**(7): 676-685.

Gomes, J. P., W. J. Bruno, M. J. Borrego and D. Dean (2004). "Recombination in the genome of Chlamydia trachomatis involving the polymorphic membrane protein C gene relative to ompA and evidence for horizontal gene transfer." J Bacteriol **186**(13): 4295-4306.

Gondek, D. C., A. J. Olive, G. Stary and M. N. Starnbach (2012). "CD4+ T cells are necessary and sufficient to confer protection against Chlamydia trachomatis infection in the murine upper genital tract." J Immunol **189**(5): 2441-2449.

Griffin, J. E., J. D. Gawronski, M. A. Dejesus, T. R. Ioerger, B. J. Akerley and C. M. Sassetti (2011). "High-resolution phenotypic profiling defines genes essential for mycobacterial growth and cholesterol catabolism." PLoS Pathog **7**(9): e1002251.

Griffiths, A. J., J. H. Miller, D. T. Suzuki, R. C. Lewontin and W. M. Gelbart (2000). An Introduction to Genetic Analysis. New York, W. H. Freeman.

Gupta, R., S. Wali, J.-J. Yu, J. P. Chambers, G. Zhong, A. K. Murthy, S. A. Bakar, M. N. Guentzel and B. P. Arulanandam (2014). "In Vivo Whole Animal Body Imaging Reveals Colonization of Chlamydia muridarum to the Lower Genital Tract at Early Stages of Infection." Molecular Imaging and Biology **16**(5): 635-641.

Hadjifrangiskou, M., A. P. Gu, J. S. Pinkner, M. Kostakioti, E. W. Zhang, S. E. Greene and S. J. Hultgren (2012). "Transposon mutagenesis identifies uropathogenic *Escherichia coli* biofilm factors." J Bacteriol **194**(22): 6195-6205.

Hamer, L., T. M. DeZwaan, M. V. Montenegro-Chamorro, S. A. Frank and J. E. Hamer (2001). "Recent advances in large-scale transposon mutagenesis." Curr Opin Chem Biol **5**(1): 67-73.

Harris, S. R., I. N. Clarke, H. M. Seth-Smith, A. W. Solomon, L. T. Cutcliffe, P. Marsh, R. J. Skilton, M. J. Holland, D. Mabey and R. W. Peeling (2012). "Whole-genome analysis of diverse *Chlamydia trachomatis* strains identifies phylogenetic relationships masked by current clinical typing." Nat Genet **44**.

Hasegawa, N., T. Sekizuka, Y. Sugi, N. Kawakami, Y. Ogasawara, K. Kato, A. Yamashita, F. Takeuchi and M. Kuroda (2017). "Characterization of the Pathogenicity of *Streptococcus intermedius* TYG1620 Isolated from a Human Brain Abscess Based on the Complete Genome Sequence with Transcriptome Analysis and Transposon Mutagenesis in a Murine Subcutaneous Abscess Model." Infect Immun **85**(2).

Hava, D. L. and A. Camilli (2002). "Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors." Mol Microbiol **45**(5): 1389-1406.

Hayes, L. J., P. Yearsley, J. D. Treharne, R. A. Ballard, G. H. Fehler and M. E. Ward (1994). "Evidence for naturally occurring recombination in the gene encoding the major outer membrane protein of lymphogranuloma venereum isolates of *Chlamydia trachomatis*." Infection and Immunity **62**(12): 5659-5663.

Hefty, P. S. and R. S. Stephens (2007). "Chlamydial type III secretion system is encoded on ten operons preceded by sigma 70-like promoter elements." J Bacteriol **189**(1): 198-206.

Hillis, S. D., L. M. Owens, P. A. Marchbanks, L. F. Amsterdam and W. R. Mac Kenzie (1997). "Recurrent chlamydial infections increase the risks of hospitalization for ectopic pregnancy and pelvic inflammatory disease." Am J Obstet Gynecol **176**(1 Pt 1): 103-107.

Hintz, N. J., D. G. Ennis, W. F. Liu and S. H. Larsen (1995). "The recA gene of Chlamydia trachomatis: cloning, sequence, and characterization in Escherichia coli." FEMS Microbiol Lett **127**(3): 175-180.

Hofreuter, D., S. Odenbreit and R. Haas (2001). "Natural transformation competence in Helicobacter pylori is mediated by the basic components of a type IV secretion system." Mol Microbiol **41**(2): 379-391.

Hooppaw, A. J. and D. J. Fisher (2016). "A Coming of Age Story: Chlamydia in the Post-Genetic Era." Infect Immun **84**(3): 612-621.

Hybiske, K. and R. S. Stephens (2007). "Mechanisms of host cell exit by the intracellular bacterium Chlamydia." Proceedings of the National Academy of Sciences **104**(27): 11430-11435.

Iliffe-Lee, E. R. and G. McClarty (1999). "Glucose metabolism in Chlamydia trachomatis: the 'energy parasite' hypothesis revisited." Molecular Microbiology **33**(1): 177-187.

Iliffe-Lee, E. R. and G. McClarty (2000). "Regulation of carbon metabolism in Chlamydia trachomatis." Mol Microbiol **38**(1): 20-30.

Ivics, Z. and Z. Izsvak (2015). "Sleeping Beauty Transposition." Microbiol Spectr **3**(2): Mdna3-0042-2014.

Jacobs, M. A., A. Alwood, I. Thaipisuttikul, D. Spencer, E. Haugen, S. Ernst, O. Will, R. Kaul, C. Raymond, R. Levy, C. R. Liu, D. Guenther, D. Bovee, M. V. Olson and C. Manoil (2003).

"Comprehensive transposon mutant library of *Pseudomonas aeruginosa*." Proceedings of the National Academy of Sciences of the United States of America **100**(24): 14339-14344.

Javed, M. A., A. J. Grant, M. C. Bagnall, D. J. Maskell, D. G. Newell and G. Manning (2010). "Transposon mutagenesis in a hyper-invasive clinical isolate of *Campylobacter jejuni* reveals a number of genes with potential roles in invasion." Microbiology **156**(Pt 4): 1134-1143.

Jeffrey, B. M., R. J. Suchland, S. G. Eriksen, K. M. Sandoz and D. D. Rockey (2013). "Genomic and phenotypic characterization of in vitro-generated *Chlamydia trachomatis* recombinants." BMC Microbiol **13**: 142.

Jeffrey, B. M., R. J. Suchland, K. L. Quinn, J. R. Davidson, W. E. Stamm and D. D. Rockey (2010). "Genome sequencing of recent clinical *Chlamydia trachomatis* strains identifies loci associated with tissue tropism and regions of apparent recombination." Infect Immun **78**(6): 2544-2553.

Johnson, C. M. and D. J. Fisher (2013). "Site-Specific, Insertional Inactivation of *incA* in *Chlamydia trachomatis* Using a Group II Intron." PLoS ONE **8**(12): e83989.

Johnston, C., B. Martin, G. Fichant, P. Polard and J.-P. Claverys (2014). "Bacterial transformation: distribution, shared mechanisms and divergent control." Nat Rev Micro **12**(3): 181-196.

Joseph, S. J., X. Didelot, J. Rothschild, H. J. de Vries, S. A. Morre, T. D. Read and D. Dean (2012). "Population genomics of *Chlamydia trachomatis*: insights on drift, selection, recombination, and population structure." Mol Biol Evol **29**(12): 3933-3946.

Joseph, S. J., H. Marti, X. Didelot, T. D. Read and D. Dean (2016). "Tetracycline Selective Pressure and Homologous Recombination Shape the Evolution of *Chlamydia suis*: A Recently Identified Zoonotic Pathogen." Genome Biol Evol **8**(8): 2613-2623.

Juhas, M. (2015). "Horizontal gene transfer in human pathogens." Crit Rev Microbiol **41**(1): 101-108.

Juretic, D., L. Zoranic and D. Zucic (2002). "Basic charge clusters and predictions of membrane protein topology." J Chem Inf Comput Sci **42**(3): 620-632.

Kapitonov, V. V. and J. Jurka (2005). "RAG1 Core and V(D)J Recombination Signal Sequences Were Derived from Transib Transposons." PLOS Biology **3**(6): e181.

Kapoor, S. (2008). "Re-emergence of lymphogranuloma venereum." J Eur Acad Dermatol Venereol **22**(4): 409-416.

Kari, L., M. M. Goheen, L. B. Randall, L. D. Taylor, J. H. Carlson, W. M. Whitmire, D. Virok, K. Rajaram, V. Endresz, G. McClarty, D. E. Nelson and H. D. Caldwell (2011). "Generation of targeted Chlamydia trachomatis null mutants." Proc Natl Acad Sci U S A **108**(17): 7189-7193.

Keravala, A., D. Liu, E. R. Lechman, D. Wolfe, J. A. Nash, D. J. Lampe and P. D. Robbins (2006). "Hyperactive Himar1 transposase mediates transposition in cell culture and enhances gene expression in vivo." Hum Gene Ther **17**(10): 1006-1018.

Kidane, D., S. Ayora, J. B. Sweasy, P. L. Graumann and J. C. Alonso (2012). "The cell pole: the site of cross talk between the DNA uptake and genetic recombination machinery." Crit Rev Biochem Mol Biol **47**(6): 531-555.

Knirsch, C. (2007). "Trachoma: ancient scourge, disease elimination, and future research." Curr Infect Dis Rep **9**(1): 21-28.

Kokes, M., J. D. Dunn, J. A. Granek, B. D. Nguyen, J. R. Barker, R. H. Valdivia and R. J. Bastidas (2015). "Integrating chemical mutagenesis and whole-genome sequencing as a platform for forward and reverse genetic analysis of Chlamydia." Cell Host Microbe **17**(5): 716-725.

Kong, Y., J. H. Ma, K. Warren, R. S. Tsang, D. E. Low, F. B. Jamieson, D. C. Alexander and W. Hao (2013). "Homologous recombination drives both sequence diversity and gene content variation in *Neisseria meningitidis*." Genome Biol Evol **5**(9): 1611-1627.

Konnyu, B., S. K. Sadiq, T. Turanyi, R. Hirmondo, B. Muller, H. G. Krausslich, P. V. Coveney and V. Muller (2013). "Gag-Pol processing during HIV-1 virion maturation: a systems biology approach." PLoS Comput Biol **9**(6): e1003103.

Koonin, E. V. (2016). "Horizontal gene transfer: essentiality and evolvability in prokaryotes, and roles in evolutionary transitions." F1000Res **5**.

Koonin, E. V., K. S. Makarova and L. Aravind (2001). "Horizontal gene transfer in prokaryotes: quantification and classification." Annu Rev Microbiol **55**: 709-742.

Krogh, A., B. Larsson, G. von Heijne and E. L. Sonnhammer (2001). "Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes." J Mol Biol **305**(3): 567-580.

Lampe, D. J., B. J. Akerley, E. J. Rubin, J. J. Mekalanos and H. M. Robertson (1999). "Hyperactive transposase mutants of the Himar1 mariner transposon." Proc Natl Acad Sci U S A **96**(20): 11428-11433.

Lampe, D. J., M. E. Churchill and H. M. Robertson (1996). "A purified mariner transposase is sufficient to mediate transposition in vitro." Embo j **15**(19): 5470-5479.

Lau, C. Y. and A. K. Qureshi (2002). "Azithromycin versus doxycycline for genital chlamydial infections: a meta-analysis of randomized clinical trials." Sex Transm Dis **29**(9): 497-502.

Lawrence, J. G. and A. C. Retchless (2009). "The interplay of homologous recombination and horizontal gene transfer in bacterial speciation." Methods Mol Biol **532**: 29-53.

Le Breton, Y., A. T. Belew, K. M. Valdes, E. Islam, P. Curry, H. Tettelin, M. E. Shirtliff, N. M. El-Sayed and K. S. McIver (2015). "Essential Genes in the Core Genome of the Human Pathogen *Streptococcus pyogenes*." Sci Rep **5**: 9838.

Le Negrate, G., A. Krieg, B. Faustin, M. Loeffler, A. Godzik, S. Krajewski and J. C. Reed (2008). "ChlaDub1 of *Chlamydia trachomatis* suppresses NF-kappaB activation and inhibits IkkappaBalpha ubiquitination and degradation." Cell Microbiol **10**.

Le Negrate, G., A. Krieg, B. Faustin, M. Loeffler, A. Godzik, S. Krajewski and J. C. Reed (2008). "ChlaDub1 of *Chlamydia trachomatis* suppresses NF-κB activation and inhibits IκBα ubiquitination and degradation." Cellular Microbiology **10**(9): 1879-1892.

Liberati, N. T., J. M. Urbach, S. Miyata, D. G. Lee, E. Drenkard, G. Wu, J. Villanueva, T. Wei and F. M. Ausubel (2006). "An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants." Proceedings of the National Academy of Sciences of the United States of America **103**(8): 2833-2838.

Long, J. E., M. DeJesus, D. Ward, R. E. Baker, T. Ioerger and C. M. Sassetti (2015). "Identifying essential genes in *Mycobacterium tuberculosis* by global phenotypic profiling." Methods Mol Biol **1279**: 79-95.

Longbottom, D. and L. J. Coulter (2003). "Animal chlamydioses and zoonotic implications." J Comp Pathol **128**(4): 217-244.

Maier, T. M., M. S. Casey, R. H. Becker, C. W. Dorsey, E. M. Glass, N. Maltsev, T. C. Zahrt and D. W. Frank (2007). "Identification of *Francisella tularensis* Himar1-based transposon mutants defective for replication in macrophages." Infect Immun **75**(11): 5376-5389.

Mandel, M. and A. Higa (1970). "Calcium-dependent bacteriophage DNA infection." J Mol Biol **53**(1): 159-162.

Marchler-Bauer, A., M. K. Derbyshire, N. R. Gonzales, S. Lu, F. Chitsaz, L. Y. Geer, R. C. Geer, J. He, M. Gwadz, D. I. Hurwitz, C. J. Lanczycki, F. Lu, G. H. Marchler, J. S. Song, N. Thanki, Z. Wang, R. A. Yamashita, D. Zhang, C. Zheng and S. H. Bryant (2015). "CDD: NCBI's conserved domain database." Nucleic Acids Res **43**(Database issue): D222-226.

Marston, A. L. and A. Amon (2004). "Meiosis: cell-cycle controls shuffle and deal." Nat Rev Mol Cell Biol **5**(12): 983-997.

Marti, H., H. Kim, S. J. Joseph, S. Dojiri, T. D. Read and D. Dean (2017). "Tet(C) Gene Transfer between Chlamydia suis Strains Occurs by Homologous Recombination after Co-infection: Implications for Spread of Tetracycline-Resistance among Chlamydiaceae." Front Microbiol **8**: 156.

Martinez, E. and F. Delacruz (1990). "Genetic Elements Involved In Tn21 Site-Specific Integration, A Novel Mechanism For The Dissemination Of Antibiotic-Resistance Genes." Embo Journal **9**(4): 1275-1281.

Matsumoto, A., H. Izutsu, N. Miyashita and M. Ohuchi (1998). "Plaque formation by and plaque cloning of Chlamydia trachomatis biovar trachoma." J Clin Microbiol **36**(10): 3013-3019.

McClarty, G. (1994). "Chlamydiae and the biochemistry of intracellular parasitism." Trends Microbiol **2**(5): 157-164.

McGrew, D. A. and K. L. Knight (2003). "Molecular Design and Functional Organization of the RecA Protein." Critical Reviews in Biochemistry and Molecular Biology **38**(5): 385-432.

Mellies, J. L., A. M. S. Barron and A. M. Carmona (2007). "Enteropathogenic and Enterohemorrhagic Escherichia coli Virulence Gene Regulation." Infection and Immunity **75**(9): 4199-4210.

Mendum, T. A., J. Newcombe, A. A. Mannan, A. M. Kierzek and J. McFadden (2011). "Interrogation of global mutagenesis data with a genome scale model of *Neisseria meningitidis* to assess gene fitness in vitro and in sera." Genome Biology **12**(12): R127-R127.

Michel, B. and D. Leach (2012). "Homologous Recombination—Enzymes and Pathways." EcoSal Plus.

Misaghi, S., Z. R. Balsara, A. Catic, E. Spooner, H. L. Ploegh and M. N. Starnbach (2006). "Chlamydia trachomatis-derived deubiquitinating enzymes in mammalian cells during infection." Mol Microbiol **61**(1): 142-150.

Mueller, K. E. and K. A. Fields (2015). "Application of beta-lactamase reporter fusions as an indicator of effector protein secretion during infections with the obligate intracellular pathogen *Chlamydia trachomatis*." PLoS One **10**(8): e0135295.

Mueller, K. E., K. Wolf and K. A. Fields (2016). "Gene Deletion by Fluorescence-Reported Allelic Exchange Mutagenesis in *Chlamydia trachomatis*." MBio **7**(1).

Mukhopadhyay, S., A. P. Clark, E. D. Sullivan, R. D. Miller and J. T. Summersgill (2004). "Detailed Protocol for Purification of *Chlamydia pneumoniae* Elementary Bodies." Journal of Clinical Microbiology **42**(7): 3288-3290.

Nair, S., S. Alokam, S. Kothapalli, S. Porwollik, E. Proctor, C. Choy, M. McClelland, S. L. Liu and K. E. Sanderson (2004). "Salmonella enterica serovar Typhi strains from which SPI7, a 134-kilobase island with genes for Vi exopolysaccharide and other functions, has been deleted." J Bacteriol **186**(10): 3214-3223.

Nelson, D. E., D. P. Virok, H. Wood, C. Roshick, R. M. Johnson, W. M. Whitmire, D. D. Crane, O. Steele-Mortimer, L. Kari, G. McClarty and H. D. Caldwell (2005). "Chlamydial IFN-gamma

immune evasion is linked to host infection tropism." Proc Natl Acad Sci U S A **102**(30): 10658-10663.

Newman, J. (2015). Characterization of Putative Cytotoxin Genes in Chlamydia Muridarum. Masters of Science Master, Indiana University.

Newton, H. J., L. J. Kohler, J. A. McDonough, M. Temoche-Diaz, E. Crabill, E. L. Hartland and C. R. Roy (2014). "A screen of Coxiella burnetii mutants reveals important roles for Dot/Icm effectors and host autophagy in vacuole biogenesis." PLoS Pathog **10**(7): e1004286.

Nguyen, B. and R. Valdivia (2014). "A chemical mutagenesis approach to identify virulence determinants in the obligate intracellular pathogen Chlamydia trachomatis." Methods Mol Biol **1197**: 347-358.

Nordstrom, K. and S. J. Austin (1989). "Mechanisms that contribute to the stable segregation of plasmids." Annu Rev Genet **23**: 37-69.

Nunes, A., M. J. Borrego and J. P. Gomes (2013). "Genomic features beyond Chlamydia trachomatis phenotypes: what do we think we know?" Infect Genet Evol **16**: 392-400.

Nunes, A. and J. P. Gomes (2014). "Evolution, phylogeny, and molecular epidemiology of Chlamydia." Infection, Genetics and Evolution **23**: 49-64.

O'Byrne, P., P. MacPherson, S. DeLaplante, G. Metz and A. Bourgault (2016). "Approach to lymphogranuloma venereum." Can Fam Physician **62**(7): 554-558.

O'Toole, G. A., L. A. Pratt, P. I. Watnick, D. K. Newman, V. B. Weaver and R. Kolter (1999). "Genetic approaches to study of biofilms." Methods Enzymol **310**: 91-109.

Ochman, H., J. G. Lawrence and E. A. Groisman (2000). "Lateral gene transfer and the nature of bacterial innovation." Nature **405**(6784): 299-304.

Omasits, U., C. H. Ahrens, S. Müller and B. Wollscheid (2013). "Protter: interactive protein feature visualization and integration with experimental proteomic data." Bioinformatics.

Osaka, I., J. M. Hills, S. L. Kieweg, H. E. Shinogle, D. S. Moore and P. S. Hefty (2012). "An automated image-based method for rapid analysis of Chlamydia infection as a tool for screening antichlamydial agents." Antimicrob Agents Chemother **56**(8): 4184-4188.

Packer, M. S. and D. R. Liu (2015). "Methods for the directed evolution of proteins." Nat Rev Genet **16**(7): 379-394.

Pal, S., W. Hui, E. M. Peterson and L. M. de la Maza (1998). "Factors influencing the induction of infertility in a mouse model of Chlamydia trachomatis ascending genital tract infection." J Med Microbiol **47**(7): 599-605.

Parrett, C. J., R. V. Lenoci, B. Nguyen, L. Russell and T. J. Jewett (2016). "Targeted Disruption of Chlamydia trachomatis Invasion by in Trans Expression of Dominant Negative Tarp Effectors." Frontiers in Cellular and Infection Microbiology **6**: 84.

Pawlikowska-Warych, M., J. Sliwa-Dominiak and W. Deptula (2015). "Chlamydial plasmids and bacteriophages." Acta Biochim Pol **62**(1): 1-6.

Pearson, W. R. (2013). "An Introduction to Sequence Similarity ("Homology") Searching." Current protocols in bioinformatics / editorial board, Andreas D. Baxevanis ... [et al.] **0 3**: 10.1002/0471250953.bi0471250301s0471250942.

Pellati, D., I. Mylonakis, G. Bertoloni, C. Fiore, A. Andrisani, G. Ambrosini and D. Armanini (2008). "Genital tract infections and infertility." European Journal of Obstetrics & Gynecology and Reproductive Biology **140**(1): 3-11.

Plasterk, R. H., Z. Izsvak and Z. Ivics (1999). "Resident aliens: the Tc1/mariner superfamily of transposable elements." Trends Genet **15**(8): 326-332.

Rajalingam, K., M. Sharma, C. Lohmann, M. Oswald, O. Thieck, C. J. Froelich and T. Rudel (2008). "Mcl-1 Is a Key Regulator of Apoptosis Resistance in Chlamydia trachomatis-Infected Cells." PLoS ONE **3**(9): e3102.

Rajaram, K., A. M. Giebel, E. Toh, S. Hu, J. H. Newman, S. G. Morrison, L. Kari and R. P. Morrison (2015). "Mutational Analysis of the Chlamydia muridarum Plasticity Zone." **83**(7): 2870-2881.

Ramadhani, A. M., T. Derrick, M. J. Holland and M. J. Burton (2016). "Blinding Trachoma: Systematic Review of Rates and Risk Factors for Progressive Disease." PLoS Negl Trop Dis **10**(8): e0004859.

Read, T. D., R. C. Brunham, C. Shen, S. R. Gill, J. F. Heidelberg, O. White, E. K. Hickey, J. Peterson, T. Utterback, K. Berry, S. Bass, K. Linher, J. Weidman, H. Khouri, B. Craven, C. Bowman, R. Dodson, M. Gwinn, W. Nelson, R. DeBoy, J. Kolonay, G. McClarty, S. L. Salzberg, J. Eisen and C. M. Fraser (2000). "Genome sequences of Chlamydia trachomatis MoPn and Chlamydia pneumoniae AR39." Nucleic Acids Res **28**(6): 1397-1406.

Rey-Ladino, J., A. G. P. Ross and A. W. Cripps (2014). "Immunity, immunopathology, and human vaccine development against sexually transmitted Chlamydia trachomatis." Human Vaccines & Immunotherapeutics **10**(9): 2664-2673.

Ridderhof, J. C. and R. C. Barnes (1989). "Fusion of inclusions following superinfection of HeLa cells by two serovars of Chlamydia trachomatis." Infect Immun **57**(10): 3189-3193.

Roberts, C. H., A. Last, S. Molina-Gonzalez, E. Cassama, R. Butcher, M. Nabicassa, E. McCarthy, S. E. Burr, D. C. Mabey, R. L. Bailey and M. J. Holland (2013). "Development and evaluation of a next-generation digital PCR diagnostic assay for ocular Chlamydia trachomatis infections." J Clin Microbiol **51**(7): 2195-2203.

Robinson, K. S., A. Clements, A. C. Williams, C. N. Berger and G. Frankel (2011). "Bax Inhibitor 1 in apoptosis and disease." Oncogene **30**(21): 2391-2400.

San Millán, R. M., I. Martínez-Ballesteros, A. Rementeria, J. Garaizar and J. Bikandi (2013). "Online exercise for the design and simulation of PCR and PCR-RFLP experiments." BMC Research Notes **6**(1): 513.

Sandoz, K. M. and D. D. Rockey (2010). "Antibiotic resistance in Chlamydiae." Future Microbiol **5**(9): 1427-1442.

Schertzer, J. W. and M. Whiteley (2011). "Microbial communication superhighways." Cell **144**(4): 469-470.

Schmitz-Esser, S., N. Linka, A. Collingro, C. L. Beier, H. E. Neuhaus, M. Wagner and M. Horn (2004). "ATP/ADP translocases: a common feature of obligate intracellular amoebal symbionts related to Chlamydiae and Rickettsiae." J Bacteriol **186**(3): 683-691.

Schoborg, R. V. (2011). "Chlamydia persistence – a tool to dissect chlamydia–host interactions." Microbes and Infection **13**(7): 649-662.

Schultz, J., F. Milpetz, P. Bork and C. P. Ponting (1998). "SMART, a simple modular architecture research tool: identification of signaling domains." Proc Natl Acad Sci U S A **95**(11): 5857-5864.

Segreti, J., K. S. Kapell and G. M. Trenholme (1992). "In vitro activity of beta-lactam drugs and sulbactam against Chlamydia trachomatis." Diagn Microbiol Infect Dis **15**(4): 371-373.

Shaw, E. I., C. A. Dooley, E. R. Fischer, M. A. Scidmore, K. A. Fields and T. Hackstadt (2000). "Three temporal classes of gene expression during the Chlamydia trachomatis developmental cycle." Molecular Microbiology **37**(4): 913-925.

Siefert, J. L. (2009). "Defining the mobilome." Methods Mol Biol **532**: 13-27.

Sota, M., M. Tsuda, H. Yano, H. Suzuki, L. J. Forney and E. M. Top (2007). "Region-specific insertion of transposons in combination with selection for high plasmid transferability and stability accounts for the structural similarity of IncP-1 plasmids." J Bacteriol **189**(8): 3091-3098.

Spratt, B. G., W. P. Hanage and E. J. Feil (2001). "The relative contributions of recombination and point mutation to the diversification of bacterial clones." Curr Opin Microbiol **4**(5): 602-606.

Sreedharan, S., M. Oram, B. Jensen, L. R. Peterson and L. M. Fisher (1990). "DNA gyrase *gyrA* mutations in ciprofloxacin-resistant strains of *Staphylococcus aureus*: close similarity with quinolone resistance mutations in *Escherichia coli*." J Bacteriol **172**(12): 7260-7262.

Stephens, R. S. (1999). Chlamydia: intracellular biology, pathogenesis, and immunity, Zondervan.

Stephens, R. S., S. Kalman, C. Lammel, J. Fan, R. Marathe, L. Aravind, W. Mitchell, L. Olinger, R. L. Tatusov, Q. Zhao, E. V. Koonin and R. W. Davis (1998). "Genome Sequence of an Obligate Intracellular Pathogen of Humans: Chlamydia trachomatis." Science **282**(5389): 754.

Stephens, R. S., S. Kalman, C. Lammel, J. Fan, R. Marathe, L. Aravind, W. Mitchell, L. Olinger, R. L. Tatusov, Q. Zhao, E. V. Koonin and R. W. Davis (1998). "Genome Sequence of an Obligate Intracellular Pathogen of Humans: *Chlamydia trachomatis*." Science **282**(5389): 754-759.

Stephens, R. S., G. Myers, M. Eppinger and P. M. Bavoil (2009). "Divergence without difference: phylogenetics and taxonomy of *Chlamydia* resolved." FEMS Immunol Med Microbiol **55**(2): 115-119.

Story, R. M., I. T. Weber and T. A. Steitz (1992). "The structure of the E. coli recA protein monomer and polymer." Nature **355**(6358): 318-325.

Suchland, R. J., A. Bourillon, E. Denamur, W. E. Stamm and D. M. Rothstein (2005). "Rifampin-resistant RNA polymerase mutants of Chlamydia trachomatis remain susceptible to the ansamycin rifalazil." Antimicrob Agents Chemother **49**(3): 1120-1126.

Suchland, R. J., K. M. Sandoz, B. M. Jeffrey, W. E. Stamm and D. D. Rockey (2009). "Horizontal transfer of tetracycline resistance among Chlamydia spp. in vitro." Antimicrob Agents Chemother **53**(11): 4604-4611.

Tam, J. E., C. H. Davis and P. B. Wyrick (1994). "Expression of recombinant DNA introduced into Chlamydia trachomatis by electroporation." Can J Microbiol **40**(7): 583-591.

Tan, M. and P. Bavoil (2012). Intracellular Pathogens I: Chlamydiales, American Society of Microbiology.

Tatusov, R. L., M. Y. Galperin, D. A. Natale and E. V. Koonin (2000). "The COG database: a tool for genome-scale analysis of protein functions and evolution." Nucleic Acids Research **28**(1): 33-36.

Taylor, H. R., M. J. Burton, D. Haddad, S. West and H. Wright (2014). "Trachoma." Lancet **384**(9960): 2142-2152.

Taylor, L. D., D. E. Nelson, D. W. Dorward, W. M. Whitmire and H. D. Caldwell (2010). "Biological characterization of Chlamydia trachomatis plasticity zone MACPF domain family protein CT153." Infect Immun **78**(6): 2691-2699.

Theobald, D. L. and S. C. Schultz (2003). "Nucleotide shuffling and ssDNA recognition in Oxytricha nova telomere end-binding protein complexes." Embo j **22**(16): 4314-4324.

Thomson, N. R., M. T. G. Holden, C. Carder, N. Lennard, S. J. Lockey, P. Marsh, P. Skipp, C. D. O'Connor, I. Goodhead, H. Norbertzcak, B. Harris, D. Ormond, R. Rance, M. A. Quail, J. Parkhill, R. S. Stephens and I. N. Clarke (2008). "Chlamydia trachomatis: Genome sequence analysis of lymphogranuloma venereum isolates." Genome Research **18**(1): 161-171.

Tipples, G. and G. McClarty (1993). "The Obligate Intracellular Bacterium Chlamydia-Trachomatis Is Auxotrophic For 3 Of The 4 Ribonucleoside Triphosphates." Molecular Microbiology **8**(6): 1105-1114.

Tjaden, J., H. H. Winkler, C. Schwoppe, M. Van Der Laan, T. Mohlmann and H. E. Neuhaus (1999). "Two nucleotide transport proteins in Chlamydia trachomatis, one for net nucleoside triphosphate uptake and the other for transport of energy." J Bacteriol **181**(4): 1196-1202.

Tusnady, G. E. and I. Simon (2001). "The HMMTOP transmembrane topology prediction server." Bioinformatics **17**(9): 849-850.

Wang, Y., S. Kahane, L. T. Cutcliffe, R. J. Skilton, P. R. Lambden and I. N. Clarke (2011). "Development of a transformation system for Chlamydia trachomatis: restoration of glycogen biosynthesis by acquisition of a plasmid shuttle vector." PLoS Pathog **7**(9): e1002258.

Wickstrum, J., L. R. Sammons, K. N. Restivo and P. S. Hefty (2013). "Conditional gene expression in Chlamydia trachomatis using the tet system." PLoS One **8**(10): e76743.

Wilkinson, A., J. Day and R. Bowater (2001). "Bacterial DNA ligases." Mol Microbiol **40**(6): 1241-1248.

Wood, D. O., R. R. Wood and A. M. Tucker (2014). "Genetic systems for studying obligate intracellular pathogens: an update." Curr Opin Microbiol **17**: 11-16.

Wright, H. R., A. Turner and H. R. Taylor (2008). "Trachoma." Lancet **371**.

Wyrick, P. B. and S. T. Knight (2004). "Pre-exposure of infected human endometrial epithelial cells to penicillin in vitro renders *Chlamydia trachomatis* refractory to azithromycin." J Antimicrob Chemother **54**(1): 79-85.

Xie, G., C. A. Bonner and R. A. Jensen (2002). "Dynamic diversity of the tryptophan pathway in chlamydiae: reductive evolution and a novel operon for tryptophan recapture." Genome Biol **3**(9): research0051.

Yahara, K., X. Didelot, M. A. Ansari, S. K. Sheppard and D. Falush (2014). "Efficient Inference of Recombination Hot Regions in Bacterial Genomes." Molecular Biology and Evolution **31**(6): 1593-1605.

Yusa, K. (2015). "piggyBac Transposon." Microbiol Spectr **3**(2): Mdna3-0028-2014.

Zaneveld, J. R., D. R. Nemergut and R. Knight (2008). "Are all horizontal gene transfers created equal? Prospects for mechanism-based studies of HGT patterns." Microbiology **154**(Pt 1): 1-15.

Zhang, D. J., H. Fan, G. McClarty and R. C. Brunham (1995). "Identification of the *Chlamydia trachomatis* RecA-encoding gene." Infect Immun **63**(2): 676-680.

Zhao, S., E. Jiang, S. Chen, Y. Gu, A. J. Shanguan, T. Lv, L. Luo and Z. Yu (2016). "PiggyBac transposon vectors: the tools of the human gene encoding." Transl Lung Cancer Res **5**(1): 120-125.

Zilsel, J., P. H. Ma and J. T. Beatty (1992). "Derivation of a mathematical expression useful for the construction of complete genomic libraries." Gene **120**(1): 89-92.

Zuck, M., A. Sherrid, R. Suchland, T. Ellis and K. Hybiske (2016). "Conservation of extrusion as an exit mechanism for *Chlamydia*." Pathog Dis **74**(7).