DEVELOPING A TOOL FOR SITE-SPECIFIC GENE INACTIVATION IN CHLAMYDIA

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DEVELOPING A TOOL FOR SITE-SPECIFIC GENE INACTIVATION IN *CHLAMYDIA*

by

Cayla Johnson

B.A., University of Missouri-Kansas City, 2011

A Thesis
Submitted in Partial Fulfillment of the Requirements for the
M.S. Molecular Biology, Microbiology and Biochemistry

Molecular Biology, Microbiology and Biochemistry Graduate Program
in the Graduate School
Southern Illinois University Carbondale
December 2014
DEVELOPING A TOOL FOR SITE-SPECIFIC GENE INACTIVATION IN CHLAMYDIA

By

Cayla Johnson

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in the field of Molecular Biology, Microbiology and Biochemistry

Approved by:

Derek Fisher, Chair

Douglas Fix

Kelly Bender

Graduate School
Southern Illinois University Carbondale
August 5, 2014
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CAYLA JOHNSON, for the Master of Science degree in Molecular Biology, Microbiology and Biochemistry, presented on AUGUST 5, 2014, at Southern Illinois University Carbondale.

TITLE: DEVELOPING A TOOL FOR SITE-SPECIFIC GENE INACTIVATION IN CHLAMYDIA

MAJOR PROFESSOR: Dr. Derek Fisher

*Chlamydia trachomatis* is an obligate intracellular bacterial pathogen that infects both humans and domestically important animals. Research in the field has, until more recently, been hindered by a lack of genetic tools. We have modified Sigma’s TargeTron™ Gene Knockout System, which utilizes a mobile group II intron for site-specific insertion and gene inactivation, for use in *C. trachomatis*. As proof of principle, we used the system to inactivate *incA*, creating mutant strains DFCT3 and DFCT4 (independent clones both carrying *incA::GII[bla]*). *IncA* is a chlamydial inclusion membrane protein involved in homotypic fusion of inclusions when cells are infected with more than one bacterium. Genotypic and phenotypic analyses were performed to ensure successful intron insertion into *incA* and loss of IncA function. Further characterization of the *incA::GII[bla]* mutant examined its pathogenicity relative to the wild type strain and indicated that the mutant was attenuated for growth in a mouse infection model, but not in a cell culture infection model. Complementation of the *incA* mutant confirmed that the phenotype differences between the wild type strain and the mutant were due to inactivation of *incA*. As *incA* mutants arise spontaneously during human infections, future work will focus on the role of IncA in pathogenesis using the mutant strains derived from this study.
ACKNOWLEDGMENTS

I would first and foremost like to thank Dr. Derek Fisher for allowing me into his lab and onto this project. His guidance and seemingly infinite patience during all of my research as well as during the writing and revision of this thesis were invaluable.

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I would be remiss to not thank my parents, William and Catherine Johnson, as well as the rest of my family, for their constant support. This includes my loyal canine companion, Olivia, for keeping me company and always being happy to see me.

Finally, I thank all of my fellow graduate students and friends for laughing, thinking, and often commiserating with me.
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Public health significance

*Chlamydia* spp. are obligate, intracellular Gram negative bacterial pathogens that are responsible for a variety of human diseases and diseases that affect economically important domestic animals (Table 1.1) (Longbottom and Livingstone, 2006). Of particular importance to human health is *Chlamydia trachomatis* (causative agent of trachoma and sexually transmitted infections [STIs]), the leading cause of reportable bacterial infections worldwide. It is also responsible for the most common reportable bacterial sexually transmitted infection in the United States (Brunham and Rappuoli, 2013). *C. trachomatis* may cause dangerous long-term complications if left untreated. In women, it increases the risk of pelvic inflammatory disease, infertility, and ectopic pregnancy (Chow *et al.*, 1990; Healy *et al.*, 1994; Paavonen and Lehtinen, 1996). In men, it can cause urethritis and lead to epididymitis and prostatitis, as well as decreased sperm viability (Mackern-Oberti *et al.*, 2013). In addition to directly causing disease, co-infection with *C. trachomatis* and human papillomavirus (HPV) puts the affected female at an increased risk for cervical cancer (Samoff *et al.*, 2005). Untreated *C. trachomatis* infection also elevates efficiency of HIV transmission (Schust *et al.*, 2012). These dangers are compounded by the frequently asymptomatic presentation of chlamydial sexually transmitted infections (Bébéar and de Barbeyrac, 2009).

*Chlamydia trachomatis* is composed of several serological variants, known as serovars. Serovars A-C cause ocular infections, which are responsible for the most
common cause of preventable blindness worldwide (Burton and Mabey, 2009).

Serovars D-K are sexually transmitted variants of *C. trachomatis*. Serovars L1-L3 cause the systemic sexually transmitted infection known as lymphogranuloma venereum (LGV) (Bébéar and de Barbeyrac, 2009).

### Table 1.1: *Chlamydia* species and diseases

<table>
<thead>
<tr>
<th>Organism</th>
<th>Host</th>
<th>Disease</th>
</tr>
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<tbody>
<tr>
<td><em>C. trachomatis</em></td>
<td>Humans</td>
<td>Trachoma, urethritis, pelvic inflammatory disease, ectopic pregnancy, lymphogranuloma venereum</td>
</tr>
<tr>
<td><em>C. pneumoniae</em></td>
<td>Humans</td>
<td>Pneumonia, bronchitis</td>
</tr>
<tr>
<td><em>C. muridarum</em></td>
<td>Hamsters, mice</td>
<td>Pneumonia</td>
</tr>
<tr>
<td><em>C. psittaci</em></td>
<td>Birds, humans</td>
<td>Psittacosis, pneumonia</td>
</tr>
<tr>
<td><em>C. abortus</em>¹</td>
<td>Ruminants, humans</td>
<td>Fetal death, abortions</td>
</tr>
<tr>
<td><em>C. suis</em>²</td>
<td>Swine</td>
<td>Conjunctivitis, pneumonia, enteritis</td>
</tr>
<tr>
<td><em>C. pecorum</em></td>
<td>Ruminants, marsupials</td>
<td>Abortion, conjunctivitis, pneumonia, infertility</td>
</tr>
<tr>
<td><em>C. felis</em>¹</td>
<td>Domestic cats</td>
<td>Conjunctivitis, rhinitis</td>
</tr>
<tr>
<td><em>C. caviae</em></td>
<td>Guinea pigs</td>
<td>Ocular inflammation</td>
</tr>
</tbody>
</table>

¹ Vaccine commercially available  
² Some strains carry the tet⁴ gene, *tet(C)*

### Bacterial growth and development

The *Chlamydia* undergo a unique biphasic developmental cycle in which the infectious, nonreplicative elementary body (EB) comes into contact with and attaches to a host cell (Figure 1.1A) (Abdelrahman and Belland, 2005). Upon contact, the EB is endocytosed where upon it resides within a host membrane-derived vesicle termed an inclusion (Figure 1.1B-C). For *Chlamydia trachomatis*, it is during the first few hours
within the inclusion that the EB will differentiate into the non-infectious, replicative reticulate body (RB) (Figure 1.1D). The RBs undergo rounds of replication via binary fission and the inclusion size increases to accommodate the growing number of bacteria. At about 18 hours post infection, the RBs begin asynchronously differentiating back into EBs (Figure 1.1E-H). Finally, at about 40 to 48 hours after infection is initiated, lysis of the inclusion and host cell allows the EBs to escape and for the infectious cycle to begin again in a new cell (Figure 1.1I). Length of the developmental cycle varies from about 40 to 72 hours depending upon the species.
Figure 1.1: *Chlamydia* developmental cycle. Modified and used with permission of Derek Fisher (Southern Illinois University).

*Chlamydia* spp. possess a type-three secretion system (T3SS) (Mueller et al., 2014). The T3SS plays an important role in modifying the inclusion at the interface of the host cytosol and inclusion membrane, a significant point for interaction between the cell and the bacterium. A group of proteins termed inclusion membranes proteins, or Incs, are secreted by the T3SS early in infection and localize to the inclusion membrane (Mital et al., 2013). There are over 50 Incs predicted within the *C. trachomatis* genome. The Incs all have a characteristic bi-lobed hydrophobic domain of 40 amino acids or
more despite having a surprising lack of sequence similarity (Figure 1.2) (Ronzone and Paumet, 2013). Currently, the function of only a small portion of the Incs is known (Table 1.2) (Agaisse and Derré, 2014; Hackstadt et al., 1999; Lutter et al., 2013; Mital et al., 2013; Rzomp et al., 2006; Scidmore and Hackstadt, 2001).

**Figure 1.2:** General Inc protein characteristics. The bi-lobed hydrophobic transmembrane (TM) region associates with the inclusion membrane while the C-terminal region, whose function is Inc-specific, is exposed to the host cytosol and allows for interaction with host components. The IncA’s SNARE-like domains enable IncA to promote homotypic fusion of multiple inclusions, as well as block host endocytic SNARE-mediated membrane fusion (Ronzone and Paumet, 2013).
Table 1.2: Known functions of *C. trachomatis* Inc proteins

<table>
<thead>
<tr>
<th><em>C. trachomatis</em> Inc proteins</th>
<th>Known biological function</th>
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<tr>
<td>IncA</td>
<td>Homotypic fusion</td>
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<tr>
<td>IncD</td>
<td>CERT recruitment</td>
</tr>
<tr>
<td>IncG</td>
<td>Adapter protein 14-3-3β recruitment</td>
</tr>
<tr>
<td>CT228</td>
<td>MYPT1 recruitment</td>
</tr>
<tr>
<td>CT229</td>
<td>Rab4 recruitment</td>
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Typically, when more than one EB infects a single host cell, the multiple inclusions will fuse until only a single inclusion is visible within the cell. IncA, a T3SS substrate, has been implicated in the process of homotypic fusion. Experiments reported by Hackstadt *et al.* used microinjections with rabbit polyclonal antibodies against three different Inc proteins into *C. trachomatis*-infected HeLa cells (Hackstadt *et al.*, 1999). The infected cells microinjected with the anti-IncA antibodies displayed multiple, non-fusing inclusions. In addition, clinical isolates have been found that lack a functional IncA protein (Suchland *et al.*, 2000). Sequencing of these isolates revealed a variety of modifications to the *incA* gene ranging from frameshift mutations to base changes resulting in early termination during translation or deletions of up to 671 base pairs (bp) leading to the production of a truncated IncA (Rockey *et al.*, 2002). Mutants also have been identified with nucleotide changes resulting in a single amino acid difference within the characteristic hydrophobic domain that significantly modifies its hydrophobicity (Suchland *et al.*, 2000). These isolates produce multiple, non-fusogenic inclusions in cells infected with more than one EB.
**Genetic tools**

Despite the public health relevance of *Chlamydia* spp., research on these pathogens has been greatly hindered by two factors: 1) their obligate, intracellular lifestyle, which has made all previous attempts at developing a system of long-term axenic growth unsuccessful and 2) a paucity of genetic tools. However, researchers in the field have recently made great strides in addressing the need for useful genetic tools and limited growth under axenic conditions has now been achieved (Agaisse and Derré, 2013; Kari *et al.*, 2011; Mishra *et al.*, 2012; Omsland *et al.*, 2012; Wickstrum *et al.*, 2013).

Within the past two years, *Chlamydia* cryptic plasmid-based expression platforms have been developed to allow for the expression of foreign and recombinant genes (Agaisse and Derré, 2013; Wickstrum *et al.*, 2013). In addition, chemical mutagenesis using ethyl methanesulfonate (EMS) has been successfully employed for forward and reverse genetic approaches, and antisense RNA has been used for gene silencing (Kari *et al.*, 2011; Mishra *et al.*, 2012). However, the field still lacked a method of targeted chromosomal gene mutation.

A mobile group II intron from *Lactococcus lactis* was previously modified for use in targeted gene disruption in prokaryotes (Lambowitz and Zimmerly, 2004). Bacterial genomes may contain both group I and group II introns. These are mobile catalytic RNA elements (Hausner *et al.*, 2014; Lambowitz and Zimmerly, 2004). The ability of mobile group II introns to splice from an RNA sequence depends on the assistance of an intron-encoded protein (IEP) that is located within the intron sequence (Lambowitz and Zimmerly, 2004). Once excised, the intron RNA is able to splice directly into the target
location within a DNA sequence. Recognition of the DNA by the intron is based on sequence similarity between the DNA and specific sites within the intron, known as the intron and exon-binding sites (IBS and EBS) (Figure 1.3A) (Lambowitz and Zimmerly, 2004). It is then reverse transcribed by the IEP, which in *L. lactis* is performed by the LtrA. Mobile group II introns share a conserved secondary structure essential for their catalytic activity despite minimal sequence similarity (Lambowitz and Zimmerly, 2004). Unlike group II introns, group I introns are self-splicing, eliminating the need for protein assistance to move from one location within the genome to another (Hausner *et al.*, 2014). Like group II introns, group I introns lack sequence similarity while retaining similar secondary structure. The insertion of group I or group II introns within an open reading frame may result in a truncated or total lack of protein if the intron does not splice out of the RNA (Saldanha *et al.*, 1993). However, since both may be spliced out, protein production and function is typically restored under wild type conditions (Hausner *et al.*, 2014; Lambowitz and Zimmerly, 2004).

To utilize the specificity and efficiency of group II introns as a mutagenesis tool, modifications were made. Unlike the wild type *L. lactis* intron, the *ltrA* ORF is removed from within the intron and placed at the 3’ end of the exon (Figure 1.3B) (Perutka *et al.*, 2004). This ensures that, upon successful intron insertion and plasmid loss, the LtrA is lost and thus unable to splice the intron from the chromosome. In place of the *ltrA* ORF, an antibiotic resistance gene is inserted to allow for mutant selection. The plasmid has an origin of replication that, ideally, allows for propagation within *E. coli*, but not within the organism in which the target gene resides, ensuring loss of the plasmid upon intron mobilization and insertion. The plasmid also contains an antibiotic resistance cassette
located outside of the group II intron to allow for selection when propagating within *E. coli* (Perutka *et al*., 2004). This system is marketed as TargeTron™ by Sigma and relies upon a proprietary algorithm that allows for retargeting of the intron, via PCR and restriction digestion, based on the sequence of the gene to be inactivated.

**Figure 1.3:** Wild type and modified *Lactococcus lactis* group II intron. (a) A simplified wild type *L. lactis* group II intron illustrates the location of the DNA recognition sites (IBS, EBS2, EBS1δ), as well as the location of the IEP (LtrA) within the intron. (b) A plasmid containing the modified group II intron in which the IEP has been moved and restriction sites added. Introns are retargeted by changing the sequence between the HindIII and BsrGI sites and “gene cargos” can be inserted at the MluI site.

This project began with an attempt to further modify this system for use in *Chlamydia* and as proof of principle we used the system for the site-specific inactivation of *incA* in *C. trachomatis* L2 434/Bu. The resulting *incA::GII(bla)* mutant displayed a
non-fusogenic phenotype when one cell was infected with multiple EBs. Using a variety of methods, the stability of the insertion was subsequently demonstrated (at both the genotype and phenotype levels) over repeated passages in cell culture and in an animal infection model in the absence of selection. More recent studies have begun to assess the pathogenicity of the mutant compared to that of the wild type parental strain using both cell culture and a mouse vaginal tract infection model. In addition, we have complemented the mutant to ensure that the growth phenotypes observed were due to the \textit{incA::GII(bla)} mutation and not a second site mutation or polar effects.

\textbf{Aims}

The goals of this project were to develop a method for targeted gene inactivation in \textit{Chlamydia} and to demonstrate the utility of this approach for studying the biology of this important pathogen.
CHAPTER 2
MATERIALS AND METHODS

Culturing conditions

Mouse fibroblast L2 cells were routinely grown at 37°C with 5% CO₂ in Dulbecco’s Modified Eagle Medium (DMEM) (HyClone, Thermo Scientific™) with GlutaMAX™ (Gibco®, Life Technologies™) supplemented with 10% fetal bovine serum (HyClone, Thermo Scientific™). PCR with degenerate primers to the Mycoplasma spp. 16s rRNA was used periodically to check the cells for mycoplasma contamination. All primers except for those listed in text are listed in Johnson and Fisher (Johnson and Fisher, 2013).

C. trachomatis was grown in L2 cells. Stocks were titered via the inclusion forming unit (IFU) assay and stored at -80°C in sucrose phosphate glycerine buffer (SPG, 0.19 mM KH₂PO₄, 0.36 mM K₂HPO₄, 0.245 mM L-glutamic acid, 10.9 mM sucrose).

E. coli strains were grown at 37°C on Luria-Bertani (LB) agar plates or in LB broth with antibiotics, as appropriate (chloramphenicol at 20 µg/mL, kanamycin at 50 µg/mL, spectinomycin at 100 µg/mL, and/or ampicillin at 100 µg/mL [all from Fisher BioReagents]).

IFU assay

Chlamydia samples were diluted in infection medium (DMEM, 10% FBS, 1× nonessential amino acids, and 0.2 µg/mL cycloheximide). 200 µl of the samples were
added per well of a black 96-well plate containing a confluent mouse L2 monolayer. To encourage simultaneous infection of cells, the plate was centrifuged at 545 × g for one hour and then incubated at 37°C for 24 hours (T₀ = start of centrifugation). After the 24 hour incubation, the infection medium was aspirated from the 96-well plate via suction and replaced with 200 µl of ice-cold methanol. The plate was incubated at room temperature for one hour or overnight at 4°C.

The methanol was removed via suction and one drop of Pathfinder™ Chlamydia Culture System (Bio-Rad) was added to each infected well and incubated at room temperature in the dark for 30 minutes. The antibody was dumped from the plate and wells were rinsed with deionized water. The plate was blotted to remove excess liquid and one drop of mounting medium was added to each well (glycerol, 10% phosphate buffered saline). Plates were either examined immediately or stored in the dark at 4°C and examined within 24 hours.

Plates were examined under a fluorescent microscope at 400× magnification under green fluorescence. Inclusions were counted in ten random fields per well and the titer determined via the following formula:

\[
\text{IFU/mL} = \frac{\text{total IFU counted}}{\text{total fields counted}} \times 233.96 \text{ fields per well} \times \text{dilution factor correction}
\]

TargeTron™ plasmid construction

The base TargeTron™ vector pACD4K-C (Sigma) was modified for use in Chlamydia by first removing the kanamycin resistance RAM cassette. The vector was digested with MluI (molecular biology reagents obtained from Thermo Scientific™ Fermentas unless noted otherwise), run on an agarose gel (using the GeneJET Gel...
Extraction and Purification Kit, Thermo Scientific™), and purified. The digested, linear plasmid lacking the cassette was then recircularized via ligation with T4 DNA ligase. The ligation was transformed into E. coli DH5α and selected on LB plates containing chloramphenicol. Colonies were then patched onto both LB agar plates containing chloramphenicol and LB agar plates containing kanamycin. A clone found to be sensitive to kanamycin, but resistant to chloramphenicol was chosen and grown for plasmid isolation (using GeneJET Plasmid Miniprep Kit, Thermo Scientific™). RAM cassette loss was confirmed via PCR using primers GIIFtest and GIIIRtest. This plasmid was designated pDFTT1.

pDFTT1 was further modified with the addition of a chlamydial promoter upstream of the GII 5’ exon. Genomic DNA from plaque-purified, clonal C. trachomatis 434/Bu L2 strain ACE051 (from Anthony Maurelli, Uniformed Services University of the Health Sciences) was used to clone the 264 bp region upstream of the CTL0655 start codon. Primers CTL0655 proF and proR were used in the PCR reaction with Platinum Taq DNA Polymerase, High Fidelity. HindIII and XbaI were used to digest both the PCR product as well as pDFTT1. After agarose gel purification, the digested PCR fragment was ligated into the digested pDFTT1. The ligation product was then transformed into E. coli DH5α and plated onto LB agar plates containing chloramphenicol to select for transformants. PCR was used to screen for the presence of the insert and a positive clone was grown for plasmid preparation before further confirmation via sequencing. This plasmid was designated pDFTT2.

The bla cassette was then moved into pDFTT2 to confer ampicillin resistance to Chlamydia upon successful intron insertion. pGFP::SW2 (gift of Ian Clarke, University of
Southampton) was used as template in a PCR reaction with primers blaF and blaR and Phusion High-Fidelity PCR Master Mix to generate a fragment containing the \textit{bla} gene. Both PCR fragment and pDFTT2 were digested with MluI, run on an agarose gel and purified. The \textit{bla}-containing fragment was then moved into pDFTT2 via ligation. Transformants were selected on LB agar plates containing both ampicillin and chloramphenicol and screened via PCR using primers blaF and GIIRtest. pDFTT2(\textit{bla}) was then sequenced for verification.

\textbf{Intron retargeting to incA}

The \textit{C. trachomatis incA} DNA sequence was submitted to the TargeTron™ Design Site and its proprietary algorithm used to determine the optimal site for intron insertion. Of the generated results, the insertion site was chosen based on the closest proximity to the 5’ start codon, as well as the lowest E-value (0.191).

Three primers were generated based on the insertion site chosen (1608|1609-IBS, 1608|1609-EBS 1d, 1608|1609-EBS2) and used along with primer EBS universal to generate a 350 bp PCR product. The product, as well as pDFTT2(\textit{bla}), was digested with HindIII and BsrGI. They were then run on an agarose gel, purified, and ligated to generate the retargeted pDFTT3. The ligation product was transformed into \textit{E. coli} DH5α and clones isolated. Primer T7 pro was then used to sequence verify the clones.

\textbf{TargeTron™ transformation and selection}

1×10^7 EBs were pipetted into a 1.5 mL microcentrifuge tube along with 3 µg pDFTT3 plasmid DNA. Molecular grade, sterile water was added to a volume of 160 µL
followed by 40 µL 5× CaCl₂. The solution was mixed by pipetting up and down 10 times and then left for 30 minutes at room temperature. After the 30 minute incubation, 33.3 µL of the transformation was added to 2 mL of ice-cold SPG for each well to be infected. This was then used to infect a confluent fibroblast L2 cell monolayer in a 6-well tissue culture plate via centrifugation for 1 hour at 545 × g. This infection is passage zero (P₀).

The mix was replaced with 2 mL DMEM/FBS and the plate incubated at 37°C with 5% CO₂.

At 12 hours post infection (T₀ = start of centrifugation), medium was replaced with 2 mL DMEM/FBS supplemented with appropriate selection antibiotic (1 µg/mL ampicillin or 200 µg/mL spectinomycin).

Between 40-44 hours post infection, P₀ was harvested. Sterile glass beads were added to the wells and the plate was rocked and swirled gently until no cells remained attached. The medium was transferred to a 50 mL conical tube and lysed via sonication with a 1/8-inch probe at 20% duty for 20 seconds. The sample was then centrifuged for 20 minutes at 4°C at 10,000 × g. The supernatant was removed and discarded and the pellet suspended in 2.5 mL SPG by vortexing and pipetting.

The P₀ harvest was then used to infect a confluent fibroblast L2 monolayer in a T75 flask. The flask was infected via rocking for two hours at 37°C with 5% CO₂ (P₁). The medium was then replaced with 20 mL of complete medium (DMEM, 10% FBS, 0.2 µg/mL cyclohexamide, 1× nonessential amino acids) with 1 µg/mL ampicillin. The flask was incubated for 40-44 hours at 37°C with 5% CO₂.

P₁ was harvested by adding sterile glass beads to the flask and rocking until all cells appeared detached. The medium was transferred to a 50 mL conical tube and
lysed via sonication at 30% duty with a 1/8-inch probe for 20 seconds. The sample was centrifuged and suspended as for P₀ harvest and used to infect P₂ as for P₁.

P₂ was harvested as described for P₁ harvest and used to infect P₃ as described above, but with an increased concentration of ampicillin at 5 µg/mL. P₃ was harvested as above and 1/3 of the harvest used to infect P₄ as an additional round of enrichment, but with ampicillin again at 1 µg/mL.

P₄ was harvested, titered via IFU assay, and used in plaque assays to obtain plaque-purified clones. Plaque assay infections and overlay feeds were done either in the presence or absence of 5 µg/mL ampicillin. Plaques were picked and expanded in the absence or presence of drug to match the conditions in which each was plaque purified. Expansion continued until each clone was used to infect eight T175 flasks which were then harvested, titered via IFU assay, and stocked at -80°C in SPG.

**Mutant genotype analysis**

Genomic DNA was purified from clones DFCT3 and DFCT4 and ACE051 using the DNeasy Blood & Tissue Kit (Qiagen) and quantified via A₂₆₀ nm absorbance using a Biotek Synergy HT microplate reader. PCR reactions were carried out using 2× PCR Master Mix (Thermo Scientific™) and primers at 0.5 µM. Genomic DNA was used at 50 ng and plasmid DNA was used at 10 ng per reaction. PCR products were run on an agarose gel and imaged via UV transillumination following ethidium bromide staining.

To sequence the insertion sites in the incA::GII(bla) clone, the ACE051, DFCT3, and DFCT4 incA regions were amplified. PCR with primers incAseqF and incAseqR was used to generate fragments containing incA that was flanked by Stul restriction
sites. The PCR products were run on an agarose gel and purified prior to StuI digestion. StuI was also used to digest pUC18. The digested PCR product was then ligated into the StuI-digested pUC18 and transformed into *E. coli* DH5α. Transformants were selected on LB agar plates containing ampicillin, which were also used for blue-white screening. Transformants were further verified by PCR and grown up for plasmid isolation. The isolated recombinant plasmids pUC18*incA*, pUC18*incA::GII(bla)*DFCT3, and pUC18*incA::GII(bla)*DFCT4 were sent for Sanger sequencing with primers GIIFtest, GIIRtest, blaF2, pUCF, and pUCR.

For Southern blot analysis, one of three enzymes (PstI, SphI, or SacI) was used to digest 2 µg of genomic DNA, which was then run on a 0.7% agarose gel. Ethidium bromide was used to stain the gels which were then viewed with a Spectrolinker™ UV transilluminator (BioRad ChemiDoc MP System). DNA was then transferred from the gel to positively charged nylon membranes. DNA was crosslinked to the membrane using a UV crosslinker set to “optimal crosslink.” The membranes were probed for DNA fragments containing *incA* or *bla* by overnight treatment with DIG-labeled DNA probes at 42°C. High-stringency washes were carried out at 65°C before probe detection. Rosche’s alkaline phosphatase anti-DIG antibody-based DIG Nucleic Acid Detection Kit was used for detection of bound probes. Addition of the precipitating chromogenic substrate nitroblue tetrazolium chloride was used to visualize bound antibody. Deionized, distilled water was added to stop the reaction. Photographs of the blots were taken using a BioRad ChemiDoc MP System.
Western blotting of IncA and MOMP

Fibroblast L2 cells were grown in a 24-well culture plate until the monolayer reached ~90% confluence. Trypsin was added to three of the wells to detach cells. Trypan blue was added to the detached cells and used for counting. A multiplicity of infection (MOI) of ten was then used to infect wells with ACE051, DFCT3, or DFCT4, diluted in 1 ml complete infection medium, via centrifugation for one hour at 545 × g at room temperature. Cells were incubated for 24 hours post infection at 37°C with 5% CO₂. A Leica DMIL inverted microscope was used to view cells. Photos were taken at 400× with a Leica EC3 camera fitted to the microscope and utilizing imaging software Leica LASV4.1 for analysis.

For Western blotting, the medium was aspirated from the wells. The wells were washed with PBS, 250 μL of 1× Laemmli buffer (containing 358 mM β-mercaptoethanol) was added, and wells were scraped with a pipet tip to harvest the cells and bacteria. Samples were transferred to Eppendorf tubes and heated for five minutes at 95°C after sonication. Samples were run in triplicate on 12% SDS-PAGE gels to allow for Coomassie Brilliant Blue staining and anti-MOMP and anti-IncA Western blotting. One gel was stained with Coomassie Brilliant Blue and imaged to ensure equal loading of the samples. The remaining gels were processed for Western blotting. The samples were transferred from the SDS-PAGE gels to nitrocellulose membranes. The membranes were blocked with 5% milk Tris-buffered saline (MTBS). Rabbit anti-IncA (gift of Dr. Raphael Valdivia, Duke University) and mouse anti-MOMP antibodies (from Abcam, gift of Wiley Jenkens, Southern Illinois University) were diluted at 1:200 and 1:1000, respectively, in MTBS and used to probe the gels overnight at 4°C. Membranes
were washed with TBS/Tween-20 (0.05% V/V). Secondary goat anti-rabbit-IgG-HRP-conjugated antibody (Thermo Scientific™ Pierce, IncA blots) or anti-mouse-IgG-HRP-conjugated antibodies (Millipore, MOMP blots) were diluted in MTBS at 1:5000 and used to probe the blots for one hour at room temperature. Blots were washed with TBS/Tween-20, then TBS, and finally treated with a chemiluminescent substrate (SuperSignal WestPico, Thermo Scientific™ Pierce). Processed blots were viewed and imaged with a BioRad ChemiDoc MP. Three independent infections were performed to assess IncA production.

Light and immunofluorescence microscopy of mutants

Fibroblast L2 monolayers in a 24-well tissue culture dishes, with some wells containing acid-treated glass coverslips, were infected as for Western blotting. Cells were infected at MOIs of 0.01 and five, for time points at 24 and 48 hours, and an MOI of ten for the 24 hour time point only. Infected replicate wells without coverslips were viewed and imaged under phase contrast microscopy at 400× magnification. For immunofluorescence (IF) microscopy, cells were fixed and permeabilized as previously described. *Chlamydia* were immunodetected by treatment first with a primary mouse anti-MOMP antibody (Abcam), then by a Texas-Red-conjugated secondary donkey-anti-mouse IgG antibody (Thermo Scientific™) and finally stained with DAPI. Coverslips were then mounted onto glass slides using ProLong Gold anti-Fade (Life Technologies™) and viewed using a Leica DM4000 fluorescent microscope. Coverslips were imaged with a QImaging QiClick Mono (QImaging) under oil immersion at 630× magnification. Images were processed with QImaging software (QImaging).
Complementation

A fragment which included wild type incA was cut from vector pUC19::incA via restriction digestion using KpnI and SalI. This fragment also contained a 47 bp upstream region in an attempt to include the incA promoter, the exact location of which is unknown. p2TK2-SW2 (gift of Isabelle Derré, Yale School of Medicine) was similarly digested with KpnI and SalI and both digested vector and incA fragment were run on a 0.7% agarose gel and purified. The incA fragment was then ligated into digested p2TK2-SW2. The ligation product was then transformed into E. coli DH5α which was grown for plasmid isolation. p2TK2-SW2::incA was sent for Sanger sequencing using primers p2tkseqF and p2tkseqR (5’-GTTCTTTCCTGCGTTAT CCC-3’; 5’- TTGAAGCGCTCCGGATAGTG-3’, respectively).

To introduce the complementation and empty p2TK2-SW2 control vectors into DFCT9, the incA::GII(aadA) mutant strain, the same chemical transformation method was used as for the TargeTron™ transformation and selection. The recombinant strain phenotypes were examined via phase contrast microscopy under 400× magnification and images taken. The subsequent recombinant strains are DFCT6 (incA::GII[aadA] p2TK2-SW2) and DFCT7 (incA::GII[aadA] p2TK2-SW2::incA).

Plaque Assays

60 mm plaque dishes with confluent L2 monolayers were infected with C. trachomatis via rocking for one hour at 37°C. 6 mL of overlay (0.5% SeaKem agarose, 1× DMEM, 10% FBS, 1× NEAA, 0.2 µg/mL cycloheximide, and antibiotics as appropriate) was added after infection and allowed to solidify for 20 minutes at room
temperature before incubation at 37°C with 5% CO₂. Plaques were fed with an additional 3 mL of overlay on days five and ten. On day 15, 1.5 mL neutral red was added to each dish and incubated for three hours at 37°C with 5% CO₂. Neutral red was then aspirated, overlay removed and discarded, and dishes allowed to dry overnight.

**Measuring plaque size**

Processed, dried plaque dishes were overturned and viewed on top of a light source. Plaques were measured at their longest point to the nearest tenth of a millimeter with a Peak Optics 7× loupe. Ten random plaques were measured per dish. Measurements were taken from duplicate plaque dishes within each trial over a series of three trials and then averaged.

**Plaque expansion**

For plaque picking, 60mm plaque dishes were infected as described above, but were not processed with the addition of neutral red after the overlay removal on day 15. 10 µL of SPG was added to a single plaque on the dish, gently scratched with a pipet tip, and pipetted up and down several times before freezing at -80°C until further expansion.

Each harvested plaque was used to infect an individual well in a confluent 24-well plate via centrifugation for one hour at 545 × g before incubation at 37°C with 5% CO₂. At 40 hours post infection, infected wells were harvested via addition of 500 µl Trition X-100 for five minutes and scraping with a pipet tip for ~20 seconds. Harvests
were centrifuged for five minutes at 13,000 × g at 4°C. Pellets were washed once with 1 mL PBS and suspended in 500 µL SPG and stored at -80°C.

**Progeny production assay**

Serial dilutions of wild type *C. trachomatis* and DFCT3 prepared in SPG were used to infect a 24-well plate with confluent L2 monolayers via centrifugation at 545 × g for one hour. 100 µL of each dilution was stored at -80°C for future titer of actual IFU input. Wells were visually observed and those with an MOI closest to 0.1 and three to five were chosen for harvest at 40 hours post infection. Harvests were performed via addition of 500 µl Triton X-100 for five minutes and scraping with a pipet tip for ~20 seconds. Harvests were centrifuged for five minutes at 13,000 × g at 4°C. Pellets were washed once with 1 mL PBS and suspended in 500 µL SPG and stored at -80°C until titering via IFU assay. Three separate infections were performed, each trial in duplicate. Harvests were titered in triplicate.

**Mouse infections**

BALB/c mice were pretreated with Depo-Provera to synchronize estrus and infected vaginally with 3×10⁶ IFU of wild type *C. trachomatis* or DFCT3 (stocks prepared and provided by D. Fisher and the author). Vaginal swabs were taken every three days from day three through day 33 post infection. Swabs were used to infect McCoy cells for titering via IFU assay. Two trials were performed and each trial had five or six mice per experimental group. Infections and titering were performed by Dr. Laxmi Yeruva (University of Arkansas for Medical Sciences).
Mouse swab expansions

Swabs were used to infect wells in a 24-well plate with a confluent L2 monolayer. Wells were harvested at 40 hours post infection via addition of 500 µL Triton X-100 for five minutes and scraping with a pipet tip for ~20 seconds. Harvests were centrifuged for five minutes at 13,000 × g at 4°C. Pellets were washed once with 1 mL PBS and suspended in 1 mL SPG and stored at -80°C until further expansion.

Harvests were used to infect L2 cells in 24-well plates and harvested as before. If inclusions could be detected visually, harvests were used to infect L2 cells in 6-well plates. Once a sufficient number of inclusions were present, EBs were harvested by addition of glass beads and hand rocking to dislodge cells. SPG was added to 1× and harvests were stored at -80°C until further processing.

The inclusion phenotype was visually observed under phase contrast light microscopy at 400× magnification once an MOI of >1 was reached. Photos were taken with a Leica LASV4 camera. The genotypes were tested using PCR on DNA purified from harvests via Qiagen DNeasy Blood & Tissue Kit.
CHAPTER 3

RESULTS

Generating an \textit{incA::GII(bla)} mutant

Mutants were obtained through chemical transformation, ampicillin selection, and isolated via plaque assay. Two plaque-purified mutants, DFCT3 and DFCT4, were chosen for additional analysis.

Mutant confirmation

It was important to examine the genotype and phenotype of the \textit{incA::GII(bla)} mutants after expansion and harvest. Intron stability in the clone expanded in the absence of selection would dictate whether or not mutants would be stable in the absence of selection during animal infection studies. Also, the desirability of this system lies in its ability to target with great specificity, making it essential to confirm its actual insertion location in the obtained clones.

Successful insertion of the intron was confirmed first through a series of PCR reactions (Johnson and Fisher, 2013). Primers were used to amplify a portion of \textit{incG}, which is located near \textit{incA} within the \textit{C. trachomatis} genome, indicating that successful insertion of GII(bla) within \textit{incA} had no effect on surrounding regions (Figure 3.1, Reaction 1). The cryptic plasmid was found to remain present in both the parental and mutant strains despite introduction of the TargeTron™ plasmid, pDFTT3 (Figure 3.1, Reaction 2). The size shift of \textit{incA} due to intron insertion was apparent when using primers that sit within \textit{incA}, flanking the insertion site (Figure 3.1, Reaction 3). Reactions
4 and 5 demonstrated the presence and orientation of the intron within \textit{incA} in the two mutant strains using two different primer sets in which one primer was located within the intron and the other within the \textit{incA} ORF (Figure 3.1). Primers specific for the intron yielded products for only the mutant strains and pDFTT3, indicating a lack of intron presence within the parental strain ACE051 (Figure 3.1, Reaction 6). Finally, primers specific for pDFTT3 only yielded product when plasmid DNA was used in the PCR reaction, indicating plasmid loss in the mutant strains after intron insertion (Figure 3.1, Reaction 7).
Figure 3.1: PCR verification of incA::GII(bla) mutants plaque-purified with or without ampicillin selection. Isolated genomic DNA from both mutant and parental strains, along with pDFTT3, were used in a series of PCR reactions to examine intron insertion, orientation, and presence or absence of cryptic and pDFTT3 plasmids. Maps of the wild type incA locus (a), incA::GII(bla) locus (c), C. trachomatis cryptic plasmid (b), and
pDFTT3 (d) show the location and expected product size of all PCR reactions performed. Figure taken from Johnson and Fisher (Johnson and Fisher, 2013).

Southern blots were performed using three different restriction enzymes and probed for *incA* and the *bla* resistance cassette. The size shift of *incA* between parental strain ACE051 and mutant strains DFCT3 and DFCT4 was apparent after probing with an *incA*-specific DNA probe. The probe-bound fragment of both mutant strains ran at a higher molecular weight than did that of the parental strain, indicating the presence of the group II intron (Figure 3.2C-E). A probe specific for the ampicillin resistance cassette (*bla*) within the intron bound to DNA fragments only present in the mutant strains. In addition, the observation of a single *bla* fragment for each mutant indicates that only a single insertion event occurred (Figure 3.2C-E).
Figure 3.2: Southern blot verification of incA size and bla presence. (a) A map of the wild type incA locus with the location of probe binding, as well as bla probe binding upon intron insertion. (b) Expected product sizes after digestion. Genomic DNA from mutant and parental strains was digested with either SacI, PstI, or SphI and run on agarose gels, stained with ethidium bromide, and viewed with UV transillumination. DNA was then transferred to a nylon membrane, probed with DIG-labeled probes against incA or bla, and detected using anti-DIG antibodies conjugated to alkaline phosphatase. Probe association was visualized with chromogenic substrate. (c-e) DNA gels are shown alongside their respective Southern blots for all enzymes. Figure taken from Johnson and Fisher (Johnson and Fisher, 2013).

Sanger sequencing was performed on pUC18 vectors carrying incA regions amplified from the parental and mutant strains. The inserts in mutant strains DFCT3 and DFCT4 had identical sequences and were also found to have inserted at the predicted location within the chromosome (position 108 within the incA open reading frame). Sequences were published by Johnson and Fisher (Johnson and Fisher, 2013).

Phenotypical analysis

Western blots confirmed the loss of IncA production. Protein samples from infected cells or mock-infected cells were run on three 12% SDS-PAGE gels. One was stained with Coomassie Brilliant Blue and imaged to confirm equal loading of samples.
(Figure 3.3A). The other two gels were processed for Western blotting: one for anti-MOMP and the other for anti-IncA antibody treatment. Treatment with anti-MOMP assured the presence of *Chlamydia* in all loaded samples (Figure 3.3B). Finally, anti-IncA antibody failed to detect any IncA protein in the samples obtained from cells infected with the *incA::GII(bla)* mutants, but did react with the ACE051 parental strain sample (Figure 3.3C). These results indicate that the GII intron insertion into *incA* on the chromosome resulted in disrupted production of IncA. Consistent with the absence of detectable IncA by Western blot, the non-fusogenic phenotype also was observed via light microscopy as well as immunofluorescence (Figure 3.4 and 3.5).

**Figure 3.3:** Western blot analysis of IncA function. Fibroblast L2 cells were infected with mutant strains, parental strain, or mock infected at an MOI of ~10. At 24 hours post infection, cells were lysed with Laemmli buffer and run on three 12% SDS-PAGE gels. One gel was stained with Coomassie Brilliant Blue (a). The other two gels were transferred to nitrocellulose membranes for anti-MOMP (b) or anti-IncA blotting (c). The predicted masses of MOMP and IncA are 42.5 kDa and 30.3 kDa, respectively. Figure taken from Johnson and Fisher (Johnson and Fisher, 2013).
Figure 3.4: IncA-null phenotype observed via phase contrast light microscopy. L2 cells were infected with parental strain ACE051 or mutant strains DFCT3 and DFCT4 at MOIs of ~10 (a-c), ~5 (j), or ~0.1 (d-i). Images were taken under 400× magnification with phase contrast microscopy at 24 (a-f) or 48 hours (g-j) post infection. Arrows indicate inclusions. Figure taken from Johnson and Fisher (Johnson and Fisher, 2013).
Figure 3.5: Immunofluorescence analysis of the incA::GII(bla) mutant phenotype.

ACE051, DFCT3, and DFCT4 were used to infect cells at an MOI of ~10. Cells were fixed at 24 hours post infection for treatment with mouse anti-MOMP (visualized with Texas Red-conjugated goat anti-mouse secondary antibody) for Chlamydia detection (b, e, h), followed by DAPI staining (a, d, g). For image overlays in panels c, f, and i,
DAPI was false colored blue and anti-MOMP was false colored red. Blue arrows indicate cell nuclei while red arrows indicate chlamydial inclusions. Images were taken at 630× magnification under oil immersion using a fluorescent microscope. Infection and immunofluorescence was carried out three times. Figure taken from Johnson and Fisher (Johnson and Fisher, 2013).

**In vitro and in vivo growth properties**

**Plaque size**

After examining the genotype and phenotype of the *incA::GII*(bla) mutant, the question arose of whether this strain would be attenuated due to lack of IncA expression. The ability to plaque was tested by measuring the size of individual plaques of the mutant strain grown under the same conditions, and measured alongside the parental strain. Plaques were measured at the longest point, from end to end. The data are representative of three separate trials in which dishes were infected in duplicate. Data were analyzed with a Student's t-test for significance (*p*-value of 0.05). The mutant strain produced plaques that were not significantly different in size than plaques produced by the parental strain (Figure 3.6A). The *incA::GII*(bla) non-fusogenic phenotype was apparent when plaque dishes were viewed at 400× magnification (Figure 3.6B).
Figure 3.6: Comparison of mutant and wild type strain plaque sizes. DFCT3 and ACE051 were used to infect cells in 60 mm plaque dishes. Dishes were incubated for two weeks and fed on days five and ten post infection. On day 14, dishes were processed and allowed to dry after overlay removal. Processed, dry plaque dishes were overturned and viewed on a light source and 10 random plaques were measured per
dish. Infections were performed in duplicate and the experiment was repeated three times. (a) Average plaque size. (b) Phase contrast micrograph of mutant plaque phenotype at 400× magnification. White arrows indicate cells containing multiple non-fusogenic inclusions. Error bars represent standard error, n=3.

**Progeny production**

In addition to plaquing ability, the *incA::GII(bla)* mutant was assessed for its ability to produce infectious progeny at numbers similar to that of the wild type strain. Progeny production was assessed *in vitro* by infecting confluent fibroblast L2 monolayers with either wild type or mutant strain at MOIs of ~0.1 and ~3-5. Infections were harvested at 40 hours post infection and titered. The harvested EBs were titered alongside a portion of the dilution used to infect that well to allow for direct comparison. The production was expressed in terms of infectious progeny harvested per EB used for infection.

When data were analyzed using a Student’s t-test (*p*-value of 0.05), the ratio of infectious progeny produced by the *incA::GII(bla)* mutant was not found to be significantly different than the parental strain (Figure 3.7). Both plaque size and progeny production results suggest that the mutant strain was not attenuated in an *in vitro* infection model compared to the wild type strain.
Figure 3.7: Wild type and mutant \textit{in vitro} progeny production. Cells were infected in a 24-well plate with wild type and mutant strains at MOIs of \( \sim 0.1 \) and three to five. Wells were harvested at 40 hours post infection via Triton X-100 and scraping. Harvests and input samples were both titered via the IFU assay. Infections were performed in duplicate and the experiment repeated three times. Error bars represent standard error, \( n=3 \).

\textbf{Animal infection model}

To determine whether the \textit{incA::GII(bla)} strain behaved similarly in an animal infection model as it did \textit{in vitro}, BALB/c mice were infected with either the wild type strain or mutant strain. Vaginal swabs were taken every three days from day three post infection through day 33. The number of infectious progeny obtained with the swabs was determined via IFU assay.
The yield in terms of IFU/mL differs from trial one to trial two, but the general trend is very similar (Figure 3.8). In both cases, the swabs taken from mice infected with the wild type strain of *C. trachomatis* contained a higher number of infectious progeny throughout the first half of the infection than did those taken from mice infected with the *incA::GII(bla)* strain. These data suggest that the mutant strain is attenuated for growth in the mouse infection model, but not in a mouse fibroblast L2 cell culture infection model.
Figure 3.8: Animal model infection. BALB/c mice were infected vaginally with wild type and mutant strains. Swabs were taken every three days from day three to day 33 post infection and titered via the IFU assay. Results from experiment one (a) and experiment two (b) with wild type in red and incA::GII(bla) mutant in blue. Five to six mice were infected per experimental group. Error bars indicate standard deviation.
Intron stability

The in vivo infection data suggest that the wild type C. trachomatis produces more infectious progeny through day 15. After day 15, the number of progeny produced by the incA::GII(bla) mutant appears much more similar to the wild type strain.

To test for the presence of the intron and maintenance of an IncA-null phenotype throughout in vivo infection, swabs taken from the mice were used to infect confluent monolayers of fibroblast L2 cells and expanded until several inclusions were visible per field under 400× phase contrast microscopy. At that point, the wells were harvested and DNA isolated. The isolated chlamydial DNA was used in PCR reactions using primers flanking the GII(bla) insertion site within incA. Figure 3.9 shows the results from swabs taken from both wild type and mutant C. trachomatis infections. The wild type swabs from days nine and twelve produced a fragment size consistent with wild type incA. The PCR product from the mutant swabs from days six and nine ran at a size indicating the presence of the GII(bla) insert (Figure 3.9A). Swabs taken from incA::GII(bla)-infected mice on days 24 and 27 were tested as those were time points in which the progeny production of the mutant approached that of the wild type strain. Again, the PCR fragments generated in this reaction ran higher than the wild type incA, indicating that the GII(bla) insert had not been lost (Figure 3.9B).
Figure 3.9: Genotype analysis to confirm the incA::GII(bla) mutation in mouse swab expansions. Swabs taken from infected mice were expanded in fibroblast L2 cells and harvested. Once detectable levels of inclusions were present, the harvests were processed for use in PCR reactions to test for GII insert presence at time points early in infection (a) and late in infection (b). Experimental sample labels indicate the day (D) on which the swab was taken, whether the mouse was from the group inoculated with the wild type (WT) or mutant (M) strain, and the mouse number.

Complementation

It was important to demonstrate that all of the observed incA::GII(bla) characteristics were due solely to the absence of IncA. Manipulation of any gene has the potential for unseen effects outside of the target gene. As a result, expression of the wild type incA gene from a plasmid resulting in restoration of the wild type phenotype can ensure that the GII(bla) insertion had no unexpected results, such as polar effects, on the expression of other genes.
Upon the fourth passage of the *incA::GII(aadA)* mutant strain carrying either p2TK2-SW2 or p2TK2-SW2::*incA* in tissue culture, the phenotype difference became apparent as every cell was infected with an MOI ~3. The strain carrying empty p2TK2-SW2 vector retained the non-fusogenic inclusion phenotype characteristic of the IncA-null mutant *C. trachomatis* (Figure 3.10A). The strain carrying the complementation vector p2TK2-SW2::*incA*, however, appeared to display a phenotype consistent with that of wild type *C. trachomatis* (Figure 3.10B). In this complemented strain, every cell appeared to contain only a single inclusion despite having an MOI >1.
Figure 3.10: Complementation of the incA::GII(aadA) mutant. Chemical transformation was used to move p2TK2-SW2 or p2TK2-SW2::incA into DFCT9. Transformants carrying p2TK2-SW2 (a) or p2TK2-SW2::incA (b) were selected with ampicillin and passaged in fibroblast L2 cells until the phenotype could be visualized. Images were taken under phase contrast microscopy at 400× magnification.
CHAPTER 4
DISCUSSION

Despite being treatable with antibiotics such as azithromycin and doxycycline, *Chlamydia trachomatis* infections continue to pose a threat to public health. Infections left untreated due to asymptomatic presentation or lack of access to antibiotics may cause a wealth of complications including blinding trachoma for ocular infections, and ectopic pregnancy, pelvic inflammatory disease, and infertility for sexually transmitted infections (Burton and Mabey, 2009; Chow *et al*., 1990; Healy *et al*., 1994; Paavonen and Lehtinen, 1996). Additionally, *Chlamydia* may enter into a persistent state in which the bacteria do not replicate, but may begin growing again when the inhibitor is removed, such as when antibiotics are no longer present (Hogan *et al*., 2004). While antibiotic resistance has not yet been seen in *C. trachomatis*, some strains of *Chlamydia suis* carry a gene that confers resistance to tetracycline (Suchland *et al*., 2009). Further, it has been shown that the tet(C) gene from *C. suis* is capable of being horizontally transferred to other species of *Chlamydia* (*C. trachomatis* and *C. muridarum*) in co-infection experiments, indicating that acquisition of antibiotic resistance genes is feasible (Suchland *et al*., 2009).

Recently, research has been done suggesting that the gastrointestinal tract may in fact be a reservoir for *Chlamydia*, resulting in long-term colonization (Yeruva *et al*., 2013b). Animals are known to become infected in the GI tract with *Chlamydia* via the fecal-oral route. Interestingly, azithromycin treatment at levels sufficient for clearing genital tract infections was found to be ineffective for clearing GI tract infections in mice (Yeruva *et al*., 2013a). Presence of *Chlamydia* in the GI tract has also been confirmed
in humans (Rank and Yeruva, 2014). Since azithromycin has been shown to be ineffective at clearing GI tract infections, individuals may still be at risk for reinfection, as well as infection of sexual partners, even after successful antibiotic treatment for genital infections.

Due to the problems associated with detection and antibiotic treatment of infections, prevention of infection would be desirable. While vaccines currently exist for C. abortus and C. felis for use in animals, vaccine development and basic research in general has been hindered due to a lack of tools for genetic manipulation and lack of axenic growth conditions, until more recently (Longbottom and Livingstone, 2006; Omsland et al., 2012). Chemical mutagenesis has been employed to introduce random chromosomal mutations, antisense RNA has been used for gene silencing, and cryptic plasmid-based expression platforms have been developed for gene expression (Agaisse and Derré, 2013; Kari et al., 2011; Mishra et al., 2012). Still, the field lacked a method of creating targeted chromosomal mutations. To address this deficiency, we modified the TargeTron™ system for use in Chlamydia to allow for targeted gene inactivation.

Use of the TargeTron™ system in Chlamydia enables researchers to target and inactivate genes without the cost and time associated with TILLING-based mutagenesis approaches. In addition, the use of multiple selection markers would enable the targeted inactivation of multiple genes. There is also potential for the study of expression when combined with the recent development of a tetracycline-inducible on/off system (Wickstrum et al., 2013). The stability of the intron makes it ideal for use in animal model infections. However, it would still be less than ideal for use in developing a live-
attenuated vaccine. In such cases, a complete deletion achieved by a method such as allelic exchange would be desirable. Despite the stability of the intron observed in our study, a true deletion eliminates any possibility of restoration of the complete wild type gene transcript.

As proof of principle, incA was targeted for inactivation because naturally-occurring IncA mutant clinical isolates have been found (Suchland et al., 2000). This indicated that insertionally inactivating the incA gene would not be lethal for the bacterium. Furthermore, C. trachomatis isolates that lack functional IncA possess a readily observable non-fusogenic inclusion phenotype. Microinjection experiments with anti-IncA supported IncA’s role in fusion of multiple inclusions in a host cell upon infection with multiple EBs (Hackstadt et al., 1999). Our creation and examination of an isogenic incA::GII(bla) mutant further supports the role of IncA in chlamydial inclusion fusion (Johnson and Fisher, 2013).

The creation of incA::GII(bla) mutants was performed in several independent trials by different members of the lab using independently-prepared EB stocks, buffers, as well as pDFTT3 plasmid DNA preparations. The mutants were first examined visually after several passages under ampicillin selection for the presence of the IncA-null phenotype. The characteristic non-fusing inclusions were apparent under light microscopy as well as immunofluorescence microscopy (Figure 3.4 and 3.5). Genotypic analysis of genomic DNA isolated from mutants DFCT3 and DFCT4 confirmed the single insertion of the GII(bla) within incA via Southern blot (Figure 3.2). PCR and sequencing determined that the insertion event occurred at the predicted location and in
the correct orientation and that pDFTT3 was lost (Figure 3.1). Finally, Western blot analysis confirmed the loss of IncA production (Figure 3.3).

Reintroduction of the *incA* gene via transformation of p2TK2-SW2::*incA* into the mutant strain resulted in the production of functional IncA (detected via Western blot, data not shown, D. Fisher) as well as restoration of the wild type fusogenic inclusion phenotype (Figure 3.10). Complementation was essential in ensuring that the mutation within *incA* did not result in any secondary mutations or polar effects in other genes. The latter point is of greater concern for genes that lie within an operon, which *incA* does not. Complementation studies have been extremely limited in *C. trachomatis* and this is only the third instance of complementation (Chen *et al*., 2014; Snavely *et al*., 2014).

After genotypic and phenotypic analysis of the *incA::GII(bla)* mutant, further characterization of the mutant was performed to examine its pathogenicity *in vivo* and *in vitro* compared to the wild type strain. Plaque dish infection assays indicated that the mutant strain produced plaques that were not significantly different in size than those of the wild type strain (Figure 3.6). However, the plaques were only measured in one dimension. Future studies should measure plaque area as the mutant plaques did show a larger-size trend and area might be a more accurate way to compare plaque size. At the very least, growth was not attenuated in the mutant strain in the plaque assays.

Production of infectious progeny was examined *in vivo* and *in vitro*. Infections of mouse fibroblast L2 cells demonstrated that the *incA::GII(bla)* mutant progeny production trend was greater than that of the wild type strain, but like the plaque results, not different in a statistically significant manner (Figure 3.7). Intriguingly, swab titers
from mouse infection experiments were higher for the wild type strain than for the mutant during the first few weeks of infection (Figure 3.8). Unlike the cell culture trends, the differences between the wild type strain and mutant strain titers in the animal model were statistically significant using repeated measures two-way ANOVA with Bonferroni correction ($p$-value of 0.05) (L. Yeruva, unpublished). Thus, the incA::GII($bla$) mutant appeared to be attenuated for growth in vivo, but not in vitro.

*C. trachomatis* clinical isolates that possess a non-fusogenic phenotype occur at a frequency of approximately 1.5%, higher than frequencies indicative of random mutations (Suchland et al., 2000). These isolates persist in clinical infection, but always at a similar frequency while the wild type continues to predominate. This implies that the wild type strain has the advantage under most conditions, but not all, or the mutant phenotype would be absent or present at considerably lower frequency. The cell culture data from our study would support an advantage for the IncA-null mutant over the wild type strain, but fails to take the innate and adaptive immune responses into account. The animal model may more accurately reflect what occurs in human infections in terms of the immune response as it appears to significantly affect the success of the mutant strain. It is possible that early response by members of the innate immune system, such as polymorphonuclear leukocytes (PMNs), to greater production of infection progeny by the mutant strain results in decreased titers compared to the wild type strain (Rank et al., 2011). Then, at later time points in which the adaptive immune response, including CD8$^+$ T-cells, respond to IncA, the wild type strain titers decrease more dramatically relative to the IncA-null strain (Wizel et al., 2002).
Initially, we hypothesized that the IncA-null strain would demonstrate attenuated growth in a cell culture infection model. We hypothesized that the presence of multiple inclusions within a single host cell would result in more competition for the scavenging of host nutrients when compared to a single, larger inclusion within a host cell. However, our data did not support this hypothesis and indicated that the exact opposite may be true. Instead of increased competition, the presence of multiple inclusions may provide a greater total surface area per bacterium across which to move nutrients from the host. This greater surface area to volume ratio would theoretically allow for more efficient nutrient uptake and could explain the increased progeny production by the mutant. This could be tested by calculating the actual surface area of the non-fusogenic inclusions within a single host cell compared to that of a single wild type inclusion.

Additionally, the increased progeny numbers could be due to greater RB production per inclusion prior to conversion to EBs. Hoare et al. have hypothesized that the triggering factor for RB to EB differentiation is decreased contact between the RB and the inclusion membrane as inclusion size increases (Hoare et al., 2008). Thus, after an EB has differentiated into an RB, it may undergo replication only as long as a certain amount of contact is maintained with the inclusion membrane. As bacterial load increases within the inclusion, it grows to accommodate them and decreases physical contact with each RB. At a certain threshold of contact, the RBs then begin differentiating back into EBs. In the context of our findings where the non-fusogenic incA::GII(bla) strain may produce more progeny than its fusogenic parental strain, this hypothesis may provide a possible explanation. The presence of multiple, smaller inclusions within the same host cell would allow more contact per RB with the inclusion
membrane. This would enable a greater number of replication events to occur before the threshold is met to signal RB to EB differentiation. This hypothesis could be tested by using the original model to determine how many RBs could be supported within the multiple non-fusogenic inclusions verses the single inclusion before they being to differentiate into EBs. This could then be tested experimentally by infecting cells with mutant and wild type strains at different MOIs to compare actual progeny production versus the calculated output based on number of inclusions.

In contrast to the cell culture infection model, the animal model infections showed that the mutant strain was attenuated for growth compared to the wild type strain during the first two weeks of infection. However, the titers of wild type and mutant strains leveled off and appeared similar during the second half of the infection, raising concerns about the stability of the intron in vivo. It was possible that the incA::GII(bla) mutant strain lost the GII(bla) insert at some point during the infection, allowing restoration of the incA gene and production of functional IncA. This would explain why the titers appeared similar for only the last half of infection. We were able to disprove this by genotypic and phenotypic analysis of the vaginal swabs taken from the infected mice. Swabs taken from the incA::GII(bla) strain-infected mice at time points late in the infection, points at which mutant and wild type strain titers became similar, still displayed the non-fusogenic mutant phenotype upon expansion in cell culture. Further, the genomic DNA isolated from those same swabs was found to have retained the intron. Thus, we concluded that similar titers of mutant and wild type strains seen late in infection were not a result of mutant strain reversion to wild type as a result of intron loss.
Another possibility is that growth of the wild type strain decreased during the second half of infection, resulting in a clearance rate equal to the mutant strain. Sera from *C. trachomatis*-infected patients have been found to contain antibodies against IncA (Bannantine *et al.*, 1998). Also, the IncA homolog found in *C. pneumoniae* is a known CD8+ T-cell antigen (Wizel *et al.*, 2002). If the adaptive immune system (which typically takes approximately ten days to develop) does in fact respond to IncA during infection, this could explain why the wild type titers from the animal infection model were reduced to levels matching those of the mutant IncA-null strain. To test this hypothesis, sera from mice infected with the wild type and mutant strains could be assessed for anti-IncA reactivity.

The *in vitro* data indicating that the mutant grows as well or better than the wild type strain were not mirrored in the results observed during the first half of the animal model. One possible explanation for the disadvantage of the incA::GII(bla) mutant strain during the first two weeks of infection *in vivo* could be an increased rate of progeny production during early infection. In a cell culture infection model, responses by the innate and adaptive immune system are absent. Thus, increased numbers may fail to elicit a response similar to what would occur *in vivo*. For early infection time points, the innate immune system would be responsible for bacterial clearance. For chlamydial infections in particular, PMNs have been found to be important for early clearance (Rank *et al.*, 2011). If this innate immune system was triggered more strongly by the presence of increased progeny numbers by the mutant during the first 48 hours of infection, the production of fewer progeny by the wild type strain might allow that strain to persist more efficiently than the mutant strain. Examination of tissue sections during
the first 24-72 hours of infection to measure PMN influx should be performed in future studies.

Collectively, this research project has resulted in the development of a tool for creating targeted chromosomal mutations in *Chlamydia* and has demonstrated the applicability of this method for delineating aspects of chlamydial pathogenesis through study of the *incA::GII* null mutant.
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