

THE ROLE OF CHLAMYDIA PROTEIN TC0600 IN GASTROINTESTINAL TRACT
INFECTION

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DEDICATION

To my parents who have been a constant source of encouragement and support during the challenges of graduate studies and life. To my wife for motivating, encouraging, and inspiring me throughout my journey of graduate studies. I am truly thankful for having all of you in my life.

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Chlamydia is the most frequently reported bacterial sexually transmitted infection in the world. Most urogenital chlamydia infections in men and women are asymptomatic, but these infections can lead to irreparable damage in the reproductive system and other tissues. Apart from the urogenital chlamydial infections, we know that chlamydia infects the gastrointestinal tract (GIT) in humans and can colonize the GIT for extended intervals without eliciting pathology. We are interested in investigating tissue tropism determinants in *Chlamydia* spp. because these could be targeted to development live-attenuated vaccines. Recently, we generated mutagenized isolates of the mouse pathogen *Chlamydia muridarum*, a close relative of the human pathogen *Chlamydia trachomatis* which causes chlamydia. One mutant that we isolated is significantly attenuated in murine gastrointestinal tissues compared to wild type, but retains its pathogenicity in the murine urogenital tract. Using novel genetic techniques, whole-genome sequencing, and complementation using newly developed vector systems we identified a chromosomal factor, *tc0600*, that we believe mediates the altered tissue tropism phenotype of this mutant in mice. Notably, the *Chlamydia trachomatis* ortholog of *tc0600* has been linked to chlamydial GIT tropism in humans.

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LIST OF ABBREVIATIONS

CT	<i>Chlamydia trachomatis</i>
LGV	Lymphogranuloma venereum
CDC	Centers for Disease Control and Prevention
MOMP	Major Outer Membrane Protein
STDs	Sexual Transmitted Diseases
WHO	World Health Organization
ITI	International Trachoma Initiative
PID	Pelvic Inflammatory Disease
MSM	Men Who Have Sex with Men
EB	Elementary Body
RB	Reticulate Body
T3SS	Type three secretion system
GIT	Gastrointestinal Tract
Inc	Inclusion Membrane
RAI	Receptive Anal Intercourse
NAAT	Nucleic Acid Amplification Testing
NG	<i>Neisseria gonorrhoeae</i>
CM	<i>Chlamydia muridarum</i>
NUG	Nongonococcal Urethritis
PZ	Plasticity Zone
ORFs	Open Reading Frames
EMS	Ethyl Methanesulfonate

PCR Polymerase Chain Reaction
SPG Sucrose Phosphate Glutamate
FBS Fetal Bovine Serum
PBS Phosphate Buffered Saline
MOI Multiplicity of Infection
PFA Paraformaldehyde
BSA Bovine Serum Albumin
aTC Anhydrotetracycline

Introduction

1.1 Background

Members of the phylum *Chlamydiae* are ancient obligate intracellular Gram-negative bacteria and can infect a wide range of hosts ranging from protozoans to humans. In the family *Chlamydiaceae*, there are three species that can cause human infection leading to disease [11]. *Chlamydia pneumoniae*, which was first isolated in 1965, can attack the human respiratory system and cause pharyngitis and pneumonia [30]. Second, *Chlamydia psittaci* normally infects birds but can also cause large outbreaks of the zoonosis psittacosis, a severe respiratory tract infection in humans [44, 47]. *Chlamydia trachomatis* (CT), the most common human chlamydial pathogen, causes trachoma, chlamydia, and lymphogranuloma venereum (LGV) [29].

Among bacterial pathogens that cause sexually transmitted diseases (STDs), CT is the most common and was recently estimated to cause 127 million infections per year, globally [45]. In last two decades in the United States, reported chlamydia cases have been increasing, with a higher rate of infection in women than in men. In 2019 alone, there were more than 1.8 million cases of chlamydia reported to US Centers for Disease Control and Prevention (CDC) [10].

Chlamydia trachomatis strains can be separated into 15 serovars (A, B, Ba, C, D, E, F, G, H, I, J, K, L1, L2 and L3) based on variation in the antigenic domains of their major outer membrane protein (MOMP). These serovars have been grouped into three biovars based on pathological outcome: trachoma (serovars A-C), chlamydia (serovars D-K), and Lymphogranuloma venereum (serovars L1-L3) [3, 22].

The trachoma biovar strains cause ocular infections and chronic conjunctivitis. Trachoma is most common in developing countries, communities with poor sanitation system, and children in these environments. These strains can be transmitted through direct contact with the nasal and/or ocular discharge of an infected person, or indirectly via fomites. Infection with the trachoma strains can lead to symptoms such as persistent conjunctivitis with eye redness, itchy eyes, and mucoid discharge. If untreated, the infection can elicit more severe symptoms such as eyelid scarring which may be followed by entropion and trichiasis. As a result, ophthalmic complications can occur such as corneal opacification, and, in some cases, non-congenital blindness [20, 29]. According to World Health Organization (WHO), in 2019 more than 95 million people received antibiotic treatment for trachoma and about 92,000 received surgical treatment [57]. Collective global efforts such as the WHO Alliances for the Global Elimination of Trachoma by 2020 and International Trachoma Initiative (ITI) have resulted in a reduction in new trachoma cases over the last few decades, and 13 countries have already reached their trachoma elimination targets [29, 56]. However, the disease continues to be a public health issue in many developing countries, and continual monitoring will be needed to avoid a rebound in trachoma cases globally.

Chlamydia is the most prevalent bacterial sexually transmitted infection and can involve the lower and upper genital tract of both sexes. In men, the chlamydia strains can cause urethritis, and more rarely, epididymitis. In contrast, most chlamydia infections in women are initially asymptomatic, but these pathogens sometimes ascend to the upper genital tract and cause severe disease. In some cases, chlamydia can cause permanent damage in the female upper genital tract, such as pelvic inflammatory disease (PID),

ectopic pregnancy, salpingitis, and infertility [20, 28, 29]. It is important to mention that in classic literature trachoma and chlamydia biovars were classified under one biovar called the ocular biovar [22]. This is because the chlamydia strains can cause neonatal conjunctivitis, which is passed from infected mothers to their infants during parturition [1]. However, the acute ocular infections caused by the chlamydia biovar strains usually do not lead to blindness or cause trachoma-like pathology.

Strains in the Lymphogranuloma venereum (LGV) biovar can cause invasive rectal and urogenital disease. Infection with the LGV strains is most prevalent in men who have sex with men (MSM), and these infections often start out, and may remain, asymptomatic. However, some patients with LGV develop proctitis, rectal ulcers, pus, and hemorrhages [53]. This pathogen can also bypass the epithelial tissues through the basolateral surface of cells and reach the draining lymph nodes which can cause lymphadenitis and permanent tissue damage [29]. People with LGV may also develop oral ulcer(s), and this might be linked with inflammation of the cervical lymph nodes [9]. LGV has also been linked to other STDs including HIV. Some studies have shown a strong association between LGV and incident HIV and vice versa [9, 53].

1.2 Chlamydial Developmental Cycle

Chlamydia spp. have a distinctive biphasic developmental cycle. This biphasic cycle is essentially a shift between two distinct forms; the elementary body (EB), metabolically inactive but highly infectious, and the reticulate body (RB), which is noninfectious and metabolically active. EBs initiate attachment and entry by binding receptors on their eukaryotic host cells. The parasitophorous vacuole within which EBs are initially endocytosed ends up becoming their unique niche within the host cell,

described as the “inclusion”. Upon entry, EBs utilize the type III secretion system (T3SS) to deliver specific protein effectors, such as Inc (inclusion membrane) proteins, that mediate inclusion maturation and other interactions with their host cells. After they form the inclusion, EBs begin transition to RBs at the mid-cycle stage, which marks the beginning of their active division phase. Towards the end of the developmental cycle, RBs begin to transform back to EBs, and the new EBs eventually exit the host cell by lysis or extrusion. Released EBs can then infect adjacent host cells, and the cycle is repeated. Importantly, during the middle of the chlamydial developmental cycle, and under circumstances such as lack of particular nutrient resources, exposure to specific antibiotics, or even intercellular stress, may cause the RBs to transition into a state of “persistence” where they remain viable but non-infectious. Once the stress is released, the RBs commence development, transform into EBs, and the canonical cycle continues [20, 22, 51, 58].

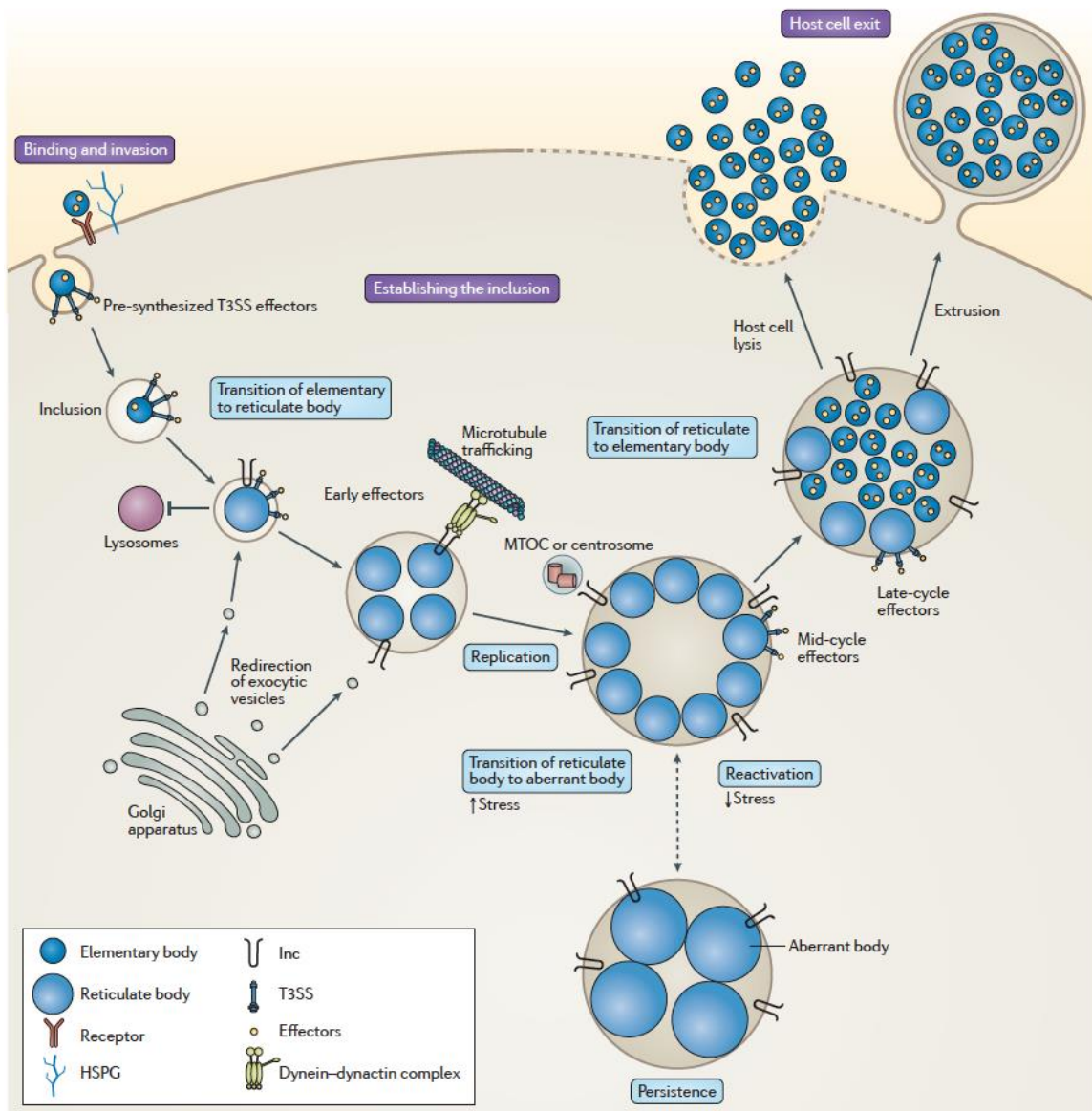


Figure 1. Chlamydial Developmental Cycle. Adapted from “Chlamydia cell biology and pathogenesis,” by C. Elwell, K. Mirrashidi, and J. Engel, 2016, NATURE REVIEWS | MICROBIOLOGY, Volume(14), 386. 2016 Macmillan Publishers Limited.

1.3 Chlamydial Gastrointestinal Tract Infection

Several studies have shown that various *Chlamydia* spp. can infect the gastrointestinal tracts (GIT) of different animals. In 1951, a study of calves by York and Baker showed that the GIT is a natural reservoir for *Chlamydia* spp. Interestingly, they reported that chlamydia can infect the intestine (cecum) of cattle for extended intervals without causing obvious symptoms [61]. Several later studies in other animals such as goats, sheep, lambs, and pigs suggested that the GIT is a normal chlamydial habitat, and also showed that various *Chlamydia* spp. can persist asymptotically in the GIT. Chlamydia transmission in most of these animals likely occurs via fecal/oral routes since grass contaminated with excrement is prevalent in the environments of these animals [44]. The duration of chlamydia gastrointestinal tract infection varies by the pathogen and host species; however, a study by Storz and Thornley in 1966 found that sheep infected with chlamydiae shed the bacteria continually for about four years [60]. The same group conducted autopsies in multiple species of infected animals, and observed that chlamydia was largely restricted to the cecum, although it was also detectable in parts of the intestines. Their findings corroborate those of York and Baker who observed that chlamydia can colonize the cecum at higher levels than in other parts of the intestines. Overall, these observations suggest that chlamydiae can colonize the GITs of many animals for extended intervals, and possibly for their lifetimes.

Multiple studies have revealed that CT can infect the GIT of both women and men. In fact, there is increasing evidence the GIT may be a reservoir for CT genital infection in women [44, 60]. In a clinical study conducted by Javanbakht and colleagues, urogenital and rectal screening was performed to detect CT and other pathogens in women who

attended an STI clinic and indicated that they had receptive anal intercourse (RAI) within 3 months prior to screening [26]. They used nucleic acid amplification testing technology (NAAT) to detect the sexually transmitted pathogens and found that rectal CT infections were more prevalent in women aged 14 to 19. Most positive cases (about 63%) were in women who had concomitant genital and rectal infections; but, 25% of CT positive results were in women who had rectal infections only. Similar to Javanbakht study, Bax and coworkers reported that about 65% of women who tested positive for CT were infected in both their rectum and cervix [6]. Dunlop et al. reported 5 cases of women who had positive rectal and urogenital cultures. These five cases were in women who were sexual contacts of men with nongonococcal urethritis (NGU) or ocular chlamydia [13, 18, 44].

In men, a clinical study conducted among MSM by Gratrix et al. detected CT in rectal and urogenital tissues [23]. Among 158 patients who tested positive for CT, 115 with a median age of 28 only had rectal infections. Most of the patients that only had rectal CT presented without any symptoms. The percentage of patients who reported RAI was higher in the men who had rectal CT, 82% and 75%, respectively. Gratrix and colleagues concluded that some of the rectal CT in the men who did not report RAI could have been linked to infections in other sites, such as the pharynx.

Many studies have documented pharyngeal CT infections, but these seem to be relatively uncommon. Peters and colleagues investigated CT and *Neisseria gonorrhoeae* (NG) in 603 women who visited a primary care clinic in South Africa [39]. They detected CT in 16% of urogenital and 7.1% of rectal specimens. Interestingly, they observed only one case of pharyngeal CT (0.2%), consistent with other studies that have found low rates

of these infections [37, 40]. Overall, the link between pharyngeal and GIT CT in humans remains unclear [4, 5].

GIT CT has been extensively documented in infants too. In 1979, a study conducted in infants by Schachter and co-workers found CT in the rectum of 4 of 12 infants aged between 40 and 80 days [48]. At that time, Schachter et al. doubted that these infections were acquired at the childbirth, because the positive culture were only detected starting at 40 days post parturition. Taylor-Robinson and Thomas suggested that the infants may have acquired these infections by a GI route via auto-inoculation with respiratory tract or conjunctival secretions, possibly because the GIT pH is higher in infants than adults [51].

1.4 Long-lasting GIT CT and Potential Consequences

Chlamydia muridarum (CM) can colonize the GIT of mice for extended intervals without causing disease [25, 38, 44]. In 2018, Wang and colleagues investigated whether CM GIT infection causes disease in mice, and they found no significant differences in GIT pathology when they compared mice that had and had not been infected with the CM Nigg strain [54]. Based on this, they suggested that CM colonization does not cause GIT disease in the colon of healthy mice. However, CM dissemination from the murine GIT has been documented. A study by Perry and Hughes showed that CM could colonize the mouse GIT for more than 8 months without causing any pathology. Interestingly, CM was detected in extra-gastrointestinal tissues such as the spleen, liver, and kidneys in the mice who were infected intranasally or orally [38]. However, Wang et al. suggested that chlamydial dissemination in this study may have been due to use of the invasive Weiss strain [43, 54]. Regardless if CM can disseminate from the murine GIT, the idea that CM

GIT infection in mice typically elicits minimal pathology or disease is well-supported [25, 60].

CT has been detected in GIT specimens from humans in many studies, and most these individuals exhibited few or no signs of GIT disease. For example, Peters et al. reported that most women who tested positive for rectal CT had no symptoms [39]. Like the Peters study, Bachmann and colleagues investigated rectal CT and NG, and found few individuals with rectal symptoms [2].

Silent GIT colonization by CT in humans is well documented. Even though LGV biovar (serovar L1-L3) can cause pathology in GIT such as proctitis in MSM [16, 22, 41]; it appears that the chlamydia biovar strains (serovar D-K) usually colonize the GIT without eliciting pathology or disease [54]. For example, Elliott et al. (1981) tested sera in total 177 patients with Crohn's disease, ulcerative colitis, and other gastrointestinal lesions, and only identified 3 individuals with antibodies to the CT chlamydia biovar (serovar D-K) [19].

Bell and co-workers reported a 2-year study in which they monitored the shedding of CT from multiple anatomic sites, including the rectum of infants born to mothers with chlamydia [8, 44]. The duration of infection in infants who cleared the infections ranged from 38 to 467 days, and from 44 to 866 days in infants who continued to show positive culture until the end of the study period. Interestingly, 6 of the 6 infants with rectal infections tested positive for CT for the entire study period, up to 380 days.

1.5 CM and Mutagenized Isolates

CM was first isolated in 1942 from respiratory tracts of asymptomatic Swiss mice [21]. This mouse pathogen, was also called *Chlamydia trachomatis*-strain mouse

pneumonitis in the seminal literature because it was considered as a discrete biovar of *Chlamydia trachomatis* [21, 22]. CM shares about 99 % of its genes with the human pathogen, *Chlamydia trachomatis* (CT) [42]. CM has been widely used in many studies in mice because it can elicit similar disease and symptoms to CT in humans. Notably, like CT in humans, CM can infect the mouse GIT without eliciting symptoms or pathology [25, 38, 60].

In *Chlamydia spp.*, there is an area of a high genomic variety called the plasticity zone (PZ) which has been hypothesized to encode chlamydial virulence and tropism genes [42]. For example, the PZ in CM encodes three putative toxins, TC0437, TC0438, and TC0439. These toxins share homology with large cytotoxins (LCTs) that contribute to *Escherichia coli* binding target cells and killing lymphocytes [7]. Rajaram et al. (2015) generated mutagenized isolates of the CM using ethyl methanesulfonate (EMS) [42]. One mutant isolate that has a nonsense mutation in *tc0439* was significantly attenuated in the mice GIT, but not the genital tract, compared to wild type (WT) CM [34]. Genome sequencing of this mutant (*tc0439*) identified an additional nonsense mutation in the *tc0600* gene. This led Morrison et al. to suggest that nonsense mutation in *tc0600* gene might mediate this tropism phenotype because the *C. trachomatis* ortholog of *tc0600* gene, *ct0326*, has also been associated with CT GI-tropism in humans [27, 34].

1.6 Research Objectives and Hypothesis

The goal of my work was to determine if mutation of the chlamydial hypothetical protein encoded by *tc0600* causes the profound attenuation of the EMS-derived CM mutant *tc0439* in the murine GIT. One aim was to investigate if *tc0600* mutants have tissue tropism defects compared to a complemented strain and wild type CM *in vitro*.

This was done by assessing if complementation with *tc0600* in trans restored the virulence the *tc0439* mutant in a cell culture model. Another goal, was to determine the localization of TC0600 in CM infected mouse cells and identify potential protein(s) it interacts with. Localization was attempted using a recombinant native and flag-tagged version of TC0600 and corresponding antibodies. A final goal of this project will be to assess if the virulence of TC0600 complemented strains is restored in a murine gastrointestinal tract model. Overall, I hypothesize that *tc0600* could be a critical determinant of CM GIT tropism in mice.

Materials and Methods

2.1 Designing the P2TK2::*tc0600* Construct

2.1.1 Amplification of *tc0600*

The *tc0600* sequence was determined from GenBank® and the gene is located between bases 715,953-717,899 in the genome of CM Strain Nigg. To amplify native *tc0600*, CM genomic DNA was isolated by adding 25 µL alkaline lysis buffer (PH=12) to 25 µL of a wild type CM EB stock (6×10^6 IFU/µL). The mixture was incubated at 90°C for 10 minutes, cooled to room temperature, and then 25 µL of neutralization buffer (1 M Tris, PH=7.5) was added. The mixture was used as template for polymerase chain reaction (PCR) amplification of *tc0600*. Primers DN-2426 and DN-2427 are described in Table 1. The dehydrated primers were reconstituted in TE buffer and stored at -20°C (concentration of 100 µ Mol). Primer annealing temperature was predicted using the NEB Tm calculator (New England Biolabs®) and confirmed by PCR using a C1000 Touch Thermal Cycler-BioRad®. The appropriate annealing temperature was determined by performing a gradient PCR experiment with a range of annealing temperatures. Total volume of the gradient PCR reaction was 20 µL. The PCR reaction components included: 9.5 µL dH₂O, 4 µL Buffer, 1 µL dNTPs, 1 µL forward-primer, 1 µL reverse-primer, 3 µL DNA template, and 0.5 µL DNA polymerase. The final PCR conditions were: 40 cycles of 98°C for 1 minute, DNA denaturation at 98°C for 30 seconds, annealing at 55-62°C for 30 seconds, extension at 72°C for 1 minute, followed by a final single extension step of 72°C for 10 minutes. After gel electrophoretic analysis, 60.7°C was selected as the annealing temperature because it yielded the cleanest amplification product. Native *tc0600* was PCR amplified in 50 µL total volume per reaction (4 reactions X 50 = 200

μL). The PCR reaction ingredients were: 27.5 μL dH₂O, 10 μL Buffer μL, 2.5 μL of dNTPs, 2.5 μL forward-primer (DN-2426), 2.5 μL revers-primer (DN-2427), 4.5 μL DNA template, and 0.5 μL of DNA polymerase enzyme (Table 1). The PCR products were purified using Molecular Biology Kit-Bio Basic® per the manufacturer protocol and were eluted in 30 μL of Elution Solution. The DNA concentration was measured using an IMPLEN NanoPhotometer®.

Primer Name	Oligonucleotide Sequence	Target
F- primer (DN2426)	‘5– GGATAACCGTATTACACCGGTG CTTGTTGGATGTGTTTGTTCGC –3’	<i>tc0600</i>
R- Primer (DN2427)	‘5– AACGGATCCGTCGACGCGGCCTT AATTTCACGACGAGAATTCTT –3’	<i>tc0600</i>

Table 1. Primers for Cloning the Native *tc0600*.

2.1.2 Preparation of Vector P2TK2

A P2TK2 plasmid stock in *E. coli* was expanded in LB broth supplemented with spectinomycin 50 μg/ mL overnight, and then the cells were pelleted by centrifugation. P2TK2 DNA was isolated from the cell pellet using a Zyppy™ Plasmid Miniprep kit, per the manufacturer instructions, and was eluted in 30 μL of Zyppy™ Elution Buffer. The DNA concentration was measured and the size of the vector was confirmed using gel electrophoresis.

Restriction digestion of P2TK2

The digestion strategy was designed using the New England Biolabs® kit and NEBcloner calculator, and the double digestion was performed in a total volume of 50 μL

containing: 1 µg vector DNA (8.3 µL of 120 ng/ µL), 5 µL (1X) of 10X NEBuffer 2.1, and 1 µL of each of the restriction enzymes KbnI and NotI-HF, and 29.7 µL of DNase/RNase-free water. The reaction was incubated 60 minutes at 37°C. The digested DNA was purified using the Molecular Biology Kit-Bio Basic flow kit, per the manufacturer instructions, and was stored at 4 °C until used in cloning experiments.

2.1.3 Cloning of *tc0600* into *P2TK2*

Fusion reaction

Cloning was performed using the NEBuilder® HiFi DNA Assembly cloning Kit (New England Biolabs®). The fusion reaction (20 µL) contained: 3 µL (total 60 ng/ µL) of linearized P2TK2, 3 µL (total 39.6 ng/ µL) of the insert DNA, 10 µL of the NEBuilder® HiFi DNA Assembly Master Mix, and 4 µL of distilled water. The reaction was mixed gently by pipetting and incubated at 50°C for 60 minutes.

Transforming the clones into competent cells

Competent cells (NEB® 5-alpha Competent *E. coli* - High Efficiency) were thawed on ice, and 2 µL of the assembly product reaction was added. The sample was mixed then was incubated on ice for 30 minutes. Control transformations were performed in parallel, including with whole P2TK2, the linearized vector, or distilled water. After 30 minutes, the samples were heat-shocked at 42°C for 30 seconds, then returned to ice for two more minutes. 950 µL of pre-warmed SOC medium was added to each transformation mixture, and then the tubes were incubated on a shaker at 250 rpm at 37°C for 60 minutes. The transformation mixture was then plated on LB agar plates supplemented with spectinomycin or chloramphenicol, as appropriate, using borosilicate beads. The plates were then incubated overnight at 37°C and then were stored at 4°C.

Confirmation of positive clones

Positive clones were identified by suspending the transformant colonies into medium and performing colony PCR. 4 µL of the colony suspensions were used as a template for the PCR reactions. The PCR reactions contained: 8.5 µL dH₂O, 4 µL Buffer µL, 1 µL of dNTPs, 1 µL forward-primer (DN-2428), 1 µL reverse-primer (DN-2429), 4 µL DNA template and 0.5 µL of polymerase (Table 2). The PCR conditions were: 98°C for 1 minute, DNA denaturation at 98°C for 30 seconds, annealing at 64°C for 30 seconds, extension at 72°C for 1 minute, for 40 cycles, followed by a final extension step at 72°C for 10 minutes. Gel electrophoresis was used to confirm that the PCR products were of the expected size.

Primer Name	Oligonucleotide Sequence	Target
F- primer (DN2428)	‘5– GACGAGCTGTACAAGTAGTTCCTCTA –3’	P2TK2
R- primer (DN2429)	‘5– GAAGAACAGCACGACAAACCCAT –3’	<i>tc0600</i>
F- primer (DN2430)	‘5– GTGCTGTTCTTCGATCTCCCAGTATC –3’	<i>tc0600</i>
R- primer (DN2431)	‘5– GGACTCCGACATCATGAAGGTCAC –3’	P2TK2

Table 2. Confirmatory Primers.

Scale up and isolation of P2TK2::*tc0600*

Four colonies that yielded PCR products of the expected size were selected for further characterization. The colonies were scaled up in 5 ml of LB broth overnight, then the plasmids were harvested from the cell pellets using the Zyppy™ Plasmid Miniprep Kit as above. The DNA concentration was measured, and gel electrophoresis was performed to confirm the size of the plasmids. The plasmids were stored at 4°C for further characterization, and glycerol stocks of the colonies that contained the plasmids were stored at – 80°C for further experiments.

Confirmation of P2TK2::*tc0600*

Clones from the previous step were confirmed using PCR to amplify either the whole *tc0600* with the P2TK2 backbone or part of the *tc0600* at the N-terminus. PCRs were performed in 20 µL using the forward primer (DN-2428) and reverse primer (DN-2431) with the same PCR ingredients and conditions as above. The second confirmatory PCR reaction was performed with forward primer (DN-2430) and reverse primer (DN-2431). Gel electrophoresis confirmed that 4 clones contained the expected inserts and the sequences of the inserts were confirmed by Sanger Sequencing.

Expand and isolate the plasmid #8 (P2TK2::*tc0600*)

The strain containing Plasmid #8 from the previous step was expanded in 50 mL of LB broth in a sterile flask, and then the cells were pelleted by centrifugation. P2TK2::*tc0600* DNA was isolated from the cell pellet using Zymo-PURE™ II Plasmid Maxiprep Kit, per the manufacturer instructions, and was eluted in 400 µL of ZymoPURE™ Elution Buffer. Concentration of the eluted DNA was measured and the DNA was stored at 4°C.

2.2 Transformation of P2TK2::*tc0600* into the *tc0439* Mutant

A transformation reaction in a total volume of 50 μL was prepared by adding DNase/RNase-free distilled water into a sterile 1.5 mL microcentrifuge tube, based upon the volume of chlamydia and the DNA in Table 3. 10 μL of 5X CaCl₂ buffer (50 mM Tris-HCL and 250 mM of CaCl₂) was added to 3 μL of the *tc0439* mutant (1.1×10^7 IFU) stock and this was mixed by pipetting. Then, 3-4 μL of P2TK2::*tc0600* plasmid (354 ng/ μL) was added (Table 3). The transformation mixture was incubated at room temperature for 30 minutes and mixed gently every 5 minutes. The mixture reaction was then transferred into 2 mL of chilled sucrose phosphate glutamate buffer (SPG) in a sterile 15 mL conical tube and was gently mixed by pipetting. The mixtures were then added to confluent mouse fibroblasts (McCoy cells) monolayers in separate wells of 6-well plates (cells were pre-seeded at 1×10^6 /well one day previously). Negative control wells received 2 mL of SPG only. The plates were spun for 60 minutes at room temperature at 1600 x g, using an eppendorf® 5810 R centrifuge, then were rocked on a thermal rocker (Thermo® Scientific) at 0.5 rpm for 30 minutes at 37°C. The SPG/transformation reaction mixture was aspirated, and 2 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) was added. The plates were incubated at 37°C, in 5% CO₂. 12 hours post-infection, the media was changed by adding 2 mL of fresh DMEM with cycloheximide & spectinomycin (50 $\mu\text{g}/\text{mL}$). The plate was incubated again for 36 hours. Then the media was removed, and the cells were harvested by adding borosilicate beads and 2 mL of chilled SPG to the wells. The SPG/EBs mixtures were centrifuged for 5 minutes at 1000 rpm to separate the cell debris and EBs. The supernatant, which contained EBs, was collected in a 2 mL sterile tube and was

stored at – 80°C until the next passage. These were then thawed on ice for about 20 minutes, and used to infect confluent monolayers of McCoy cells in 6-well plates. The passage followed the same steps above, except that the cycloheximide & spectinomycin were added to the DMEM media directly. These steps were repeated for passage 1, passage 2, and passage 3. At passage 3 (P3), and after incubating the plate for about 24 hours, the mCherry positive inclusions were viewed using a microscope (AMG EVOS FL Fluorescence Imaging Microscope) using a filter for Texas Red (10x and 20x). Positive wells were harvested and passaged again for P4, similarly as above. Finally, EBs were harvested from P4 and were stored at – 80°C.

Reaction	dH2O	CaCl2	<i>tc0439</i> mutant	DNA
#1	34 µL	10 µL	3 µL of 1.1X10 ⁷ IFU	3 µL of 354 ng/ µL
#2	33 µL	10 µL	3 µL of 1.1X10 ⁷ IFU	4 µL of 354 ng/ µL

Table 3. Transformation Reactions for the Native *tc0600* into *tc0439* Mutant.

2.3 Plaque Assay

A tube of passage 4 from the previous step was thawed on ice for 20 minutes. 10 µL was added into 90 µL of SPG in an 1.5 eppendorf tube, and a 10-fold dilution series to 10⁻⁶ was prepared in cold SPG. 10 µL was taken from each dilution and added into 2 mL of SPG in a 15 mL conical tube. The SPG/EBs for each dilution were mixed and then placed into a well of a 6-well plate that contained a McCoy cell monolayer. The plate was centrifuged at 1600 x g for 60 minutes, then rocked at 37°C for 30 minutes at 0.5 rpm. The SPG/EBs mixture was aspirated, and then a mixture of 1 mL of DMEM (with 20 µL cycloheximide & 20 µL of spectinomycin) and 1 mL of pre-heated liquid agarose were

mixed and poured into each well. After the agarose solidified, 2 mL DMEM supplemented with 20 μ L cycloheximide and 20 μ L of spectinomycin was overlaid on the agarose plug in each well. The plates were incubated for 48 hours at 37°C, 5% Co₂. After 48 hours, the media was replaced, and the plate was incubated for 48 hours at 37°C, 5% Co₂. On day 6, infected zones in the well were observed and marked from the bottom of the plate. These were then picked using wide-bore pipette tips and the individual plaque plugs were added to 120 μ L of chilled SPG in 1.5 eppendorf sterile tubes with 3 borosilicate beads. The tubes were placed in a eppendorf® Thermomixer R at 4°C and agitated for 10 minutes at 100 rpm. The plaque lysates were stored at – 80°C.

2.4 Scale-Up of the Purified Colonies

Plaque lysate tubes were thawed on ice for 20 minutes. 100 μ L from each tube was added to 2 ml SPG in a conical tube. The SPG/EBs mixture were used to infect individual wells of 6-well plates with McCoy well monolayers, as in the previous section. 48 hours post infection, the wells were harvested using 2 mL of chilled SPG as in the transformation protocol. EBs supernatants were collected in 1.5 eppendorf tubes and stored at – 80°C.

2.5 Expand the Complement Via Large Chlamydia Prep

4 flasks of T175 seeded were with McCoy cells. The flasks were infected by EBs from the second scale up (step 2.4). Infection was performed by adding EBs from the second scale-up to 20 mL of SPG. The SPG/EBs were mixed and added to a T175 flask from which the media had been aspirated. The flasks were rocked at 37°C for 2 hours at 0.5 rpm. Then the infection of SPG/EBs mixture was aspirated, and 22 ml of DMEM plus 220 μ L cycloheximide and 220 μ L of spectinomycin was added. The flasks were

incubated for 48 hours at 37°C, 5% CO₂. The media was aspirated, and the EBs were harvested using borosilicate beads and chilled SPG. The harvested EBs were purified using the Chlamydia Mid-Large-Scale Preparation protocol from the Caldwell lab.

2.6 Transformation of P2TK2 into *tc0439*

Empty P2TK2 was also transformed into *tc0439*. The P2TK2::*tc0600* plasmid from section 2.2 was used as a positive control for these experiments. The transformation reactions are described in Table 4. Transformants that were obtained were characterized similarly to the P2TK2::*tc0600* transformant.

Reaction	dH ₂ O	CaCl ₂	<i>tc0439</i> mutant	DNA
#1	33 µL	10 µL	3 µL of 1.1X10 ⁷ IFU	4 µL of 306 ng/ µL
#2	33 µL	10 µL	3 µL of 1.1X10 ⁷ IFU	4 µL of 193 ng/ µL
#3	33 µL	10 µL	3 µL of 1.1X10 ⁷ IFU	4 µL of 113 ng/ µL
#4	33 µL	10 µL	3 µL of 1.1X10 ⁷ IFU	4 µL of 122 ng/ µL
Positive control	33 µL	10 µL	3 µL of 1.1X10 ⁷ IFU	4 µL of 354 ng/ µL

Table 4. Transformation Reactions for Empty Vector into *tc0439* Mutant.

2.7. Cloning of *tc0600*::1xFLAG into P2TK2

Following a similar protocol for cloning of native *tc0600* into P2TK2 (step 2.1), FLAG-tag *tc0600* constructs were made using primers DN-2450 and DN-2451 (Table 5). The final construct was confirmed by PCR and sequencing, using the primers in Table 2, similarly to the native *tc0600* clone.

Primer Name	Oligonucleotide Sequence	Target
F- primer (DN2450)	‘5– CCGTATTACACCGGTGGTACCAAA GTAATAGACGGAGGATAGGACA –3’	<i>tc0600</i>
R- primer (DN2451)	‘5– GGATCCGTCGACGCGGCCGCTTACTT ATCGTCGTCATCCTTGTAATCATTCCA CGACGAGAATTTCTTACC –3’	<i>tc0600</i>

Table 5. Primers for Obtaining *tc0600::1xFLAG* Construct.

2.8 Transformation of *tc0600::1xFLAG* into *tc0439*

P2TK2::*tc0600::1xFLAG* plasmid was transformed into *tc0439* following the same transformation protocol described above. Transformation of 6 plasmid isolates from 6 different clones was attempted. Two positive control transformations were performed in a separate 6-well plate and were discarded when they displayed positive results at passage 3 to avoid risk of cross-contamination. The transformation conditions are in Table 6.

Reaction	dH2O	CaCl2	<i>tc0439</i> mutant	DNA
#1	28.3 µL	10 µL	3 µL of 1.1X10 ⁷ IFU	8.7 µL of 115 ng/ µL
#2	29.0 µL	10 µL	3 µL of 1.1X10 ⁷ IFU	8.0 µL of 124 ng/ µL
#3	28.2 µL	10 µL	3 µL of 1.1X10 ⁷ IFU	8.8 µL of 113 ng/ µL
#4	27.3 µL	10 µL	3 µL of 1.1X10 ⁷ IFU	9.7 µL of 103 ng/ µL
#5	29.0 µL	10 µL	3 µL of 1.1X10 ⁷ IFU	8.0 µL of 124 ng/ µL
#6	32.9 µL	10 µL	3 µL of 1.1X10 ⁷ IFU	4.1 µL of 245 ng/ µL
Positive control	34.5 µL	10 µL	3 µL of 1.1X10 ⁷ IFU	2.5 µL of 410 ng/ µL
Positive control	34.7 µL	10 µL	3 µL of 1.1X10 ⁷ IFU	2.3 µL of 440 ng/ µL

Table 6. Transformation Reactions for P2TK2::*tc0600::1xFLAG* into *tc0439*.

2.9 Transformation of *tc0600::1xFLAG* into Wild Type CM

The P2TK2::*tc0600::1xFLAG* plasmids were transformed into WT CM as described for other plasmids using similar positive and negative controls. The transformation conditions are described in Table 7.

Reaction	dH2O	CaCl2	WT CM	DNA
#1	26.0 µL	10 µL	6 µL of 6X10 ⁶ IFU	8.0 µL of 124 ng/ µL
#2	25.3 µL	10 µL	6 µL of 6X10 ⁶ IFU	9.7 µL of 103 ng/ µL
#3	26.0 µL	10 µL	6 µL of 6X10 ⁶ IFU	8.0 µL of 124 ng/ µL
#4	23.0 µL	10 µL	6 µL of 6X10 ⁶ IFU	11.1 µL of 90 ng/ µL
#5	21.5 µL	10 µL	6 µL of 6X10 ⁶ IFU	12.5 µL of 80 ng/ µL
#6	25.0 µL	10 µL	6 µL of 6X10 ⁶ IFU	9.0 µL of 110 ng/ µL
Positive control	31.5 µL	10 µL	6 µL of 6X10 ⁶ IFU	2.5 µL of 410 ng/ µL
Positive control	31.7 µL	10 µL	6 µL of 6X10 ⁶ IFU	2.3 µL of 440 ng/ µL

Table 7. Transformation Reactions for P2TK2::*tc0600*::1xFLAG into wt CM.

2.10 Polyclonal Antibodies Against *tc0600*

We contracted the production of polyclonal antibodies (pAbs) against TC0600 protein epitopes to Sino Biological. The sequences of peptides were determined from the *tc0600* gene based on antigenic analysis. Peptide #1A is the native sequence, and since it has 3 residues of cysteine, two were replaced with serine residues to avoid improper conjugation, to yield the peptide #1B (Table 8). Immunization of rabbits was performed using conjugated peptide #1A and Peptide #2. All of these peptides were used for evaluating the final titer. The antibodies were purified from convalescent sera using Peptide #1B and Peptide #2.

Peptide Name	Sequence
Peptide #1A	PKP <u>S</u> SN <u>D</u> S <u>N</u> QNEGC (mutated)
Peptide #1B	PKPC <u>S</u> ND <u>C</u> NQNEGC (native)
Peptide #2	QSTAERVDSVISD-C

Table 8. Sequences of Antigenic Peptides.

2.11 Cell Culture Methods

I used 2 cell lines in this project: mouse fibroblasts cells (McCoy) and mouse epithelial cells (CMT-93). The McCoy and CMT-93 lines were recovered at passage 6-7 and were used between passage 9 and passage 20 in experiments. The cell lines were maintained in T75 flasks and split every 48 hours using the following protocol. The media was aspirated from the flask and the cells were washed with 8 mL of phosphate buffered saline (PBS). The PBS was aspirated, and then 1 mL of 1X trypsin was added to the cells. The flask was incubated for 5-10 minutes at 37 °C. 4 mL of DMEM supplemented with 10% FBS was then added and was mixed with trypsinized cells. 1.5 mL of this mixture was combined with 13 mL of DMEM supplemented with 10% FBS. The flask was then incubated for 48 hours at 37°C, 5% Co₂, until the next passage.

2.12 Immunofluorescent Assay Analysis for Visualizing and Measuring Inclusions

Monolayers of infected cells were fixed with methanol for 5 minutes, then were washed 3X with PBS. The monolayers were labeled with the primary antibody E6H1, diluted 1:10 in PBS, for 60 minutes. Then, the cells were washed with PBS 3X. Next, the cells were labeled with a 1:1000 dilution of anti-mouse Abs (DyLight™ 488 goat, anti-mouse IgG isotype: goat polyclonal) in PBS for 60 minutes in the dark. The cells were

then washed again 3X with PBS. Finally, the plates were imaged using BioTek® CYTATION 5, and inclusions numbers and sizes were calculated.

2.13 IFA Analysis of TC0600 Expression

Chlamydia infected monolayers were fixed with methanol as described above, or in 4% of paraformaldehyde (PFA) for 15 minutes. The methanol fixed wells were labeled with E6H1 or various dilutions of polyclonal Anti-TC0600 antibodies, similarly as above. The PFA fixed cells were labeled similarly, except that the labeling was performed in IFA buffer (1% bovine serum albumin (BSA), 0.1% saponin in PBS) for 45 minutes after fixation, and then were washed 3X with PBS, and were labeled with primary antibodies overnight.

Cells labeled with the control primary Abs (E6H1), were labeled with the secondary 1:1000 anti-mouse Abs (DyLight™ 488 goat, anti-mouse IgG isotype: goat polyclonal). Cells labeled with primary polyclonal Anti-TC0600 antibodies were labeled with secondary 1:1000 anti-rabbit Abs (Donkey anti-rabbit IgG, isotype: Donkey polyclonal). Both of the incubations were performed for 60 minutes in dark. After the incubation, the cells were washed 3X with PBS.

2.14 Phenotyping *tc0439* in vitro

Plates were seeded with McCoy & CMT-93 cells and infected with the same infection protocol mentioned for the IFA experiments. 24 hours post-infection, the plates were fixed with the methanol for 5 minutes and washed 3X with PBS. The cells were labeled with 1:10 E6H1 for 60 minutes, then were washed 3X with PBS. Cells were then labeled with the 1:1000 secondary anti-mouse Abs and incubated for 60 minutes in dark. After the incubation, the wells washed 3X with the PBS.

2.15 Immunoblotting

Cell monolayers infected with various chlamydia strains were harvested by adding 400 μ L of Pierce™ RIPA Buffer (Thermo® Scientific), and the cells were collected using sterile scrapers. The lysates were placed into a 1.5 mL eppendorf tube on ice. The tubes were centrifuged at 14000 rpm for 15 minutes at 4°C. The supernatant was then transferred to a new 1.5 mL tube on ice. The protein samples were stored at -20°C.

Protein concentration in the samples were determined using the Bradford protein assay and corrected to 25 μ g/mL. Protein samples were thawed on ice and mixed with the loading buffer (4X KALB) in a sterile 1.5 mL eppendorf tube. The tubes were boiled at 95°C for 5 minutes, cooled to room temperature, and centrifuged at 14000 rpm for 5 minutes to isolate the protein supernatants. A 12 well gel (10% Mini-PROTEAN® TGX™ Precast Gels) was placed in the electrode chamber inside the electrophoresis tank. 8 μ L of GE Rainbow Full Range marker was loaded in wells number 1 and 8. Electrophoresis was performed at 65V for 90 minutes. The gel was then placed into a tray filled with transfer buffer. The proteins were transferred from the gel to nitrocellulose or polyvinylidene fluoride (PVDF) membranes at 100V for 60 minutes. Next, the membranes were blocked in 5% blocking buffer for 60 minutes on a rocker at 1 rpm at room temperature.

The membranes were labeled with primary Abs 1:2000 GAPDH in blocking buffer, or 1:50 polyclonal Anti-TC0600 in the blocking buffer, overnight at 4°C. The membranes were washed 3X with tris-buffered saline and Tween 20 (TBST) for 10 minutes. The membranes labeled with GAPDH were labeled with secondary 1:5000 Anti-mouse IgG, HRP-linked antibody in blocking buffer, whereas the membranes labeled

with Anti-TC0600 antibodies were labeled with a secondary 1:1500 Anti-rabbit IgG, HRP-linked antibody in blocking buffer, for 60 minutes in both cases. After incubation with the secondary Abs, the membranes were washed 3X with TBST (10 minutes for each). The membranes were developed on plastic wrap by adding 1:1 of SuperSignal™ West Pico PLUS Luminol/Enhancer and SuperSignal™ West Pico PLUS Stable Peroxide (400 µL each) and incubating 10 minutes at room temperature. The excess reagent was removed, and the blots were covered with plastic wrap. Finally, the blots were imaged in the dark for 10 minutes using a BioRad® Molecular Imager–ChemiDoc XRS+ imaging system. A similar procedure was used for blotting of the strains carrying P2TK2::*tc0600*::1xFLAG, except that a primary 1:500 Anti-FLAG M2 (Sigma) antibody was used and the secondary antibodies was a 1:4000 Anti-mouse IgG, HRP-linked antibody.

Results

3.1 The Size of TC0600 Complemented and Vector Only Strains are Similar

To study the role of the CM protein Tc0600 in CM colonization of the murine GIT, we decided to test if the phenotypes of *tc0600* mutants were reversed by complementation with native and recombinant versions of Tc0600. Complemented strains were obtained using a transformation protocol adapted from Wang et al. (2011) and Cortina et al. (2019) [55, 12] with some modifications. Overall, chemical transformation using calcium chloride (CaCl₂) has been a very effective approach for stable introduction of other recombinant plasmids into chlamydiae. We were able to successfully construct transformants in which we introduced native *tc0600* into the *tc0439* mutant, the empty vector (P2TK2) into the *tc0439* mutant, *tc0600::1xFLAG* into the *tc0439* mutant, and *tc0600::1xFLAG* into wt CM.

The different transformation experiments yielded positive results at different passages, and the sizes of the transformant inclusions varied from experiment to experiment. For example, native *tc0600* and the empty vector yielded transformants between passage 2 and passage 3. In contrast, P2TK2::*tc0600::1xFLAG* transformants of the *tc0439* mutant were only detected at late passages 5 & 6, even though five out of six attempts to construct these transformants were eventually successful (Table 5). In wt CM, three transformation experiments succeeded. Notably, these transformants were only detected between passages 5 and 7. Interestingly, one plasmid (#3) was recovered at passage 3 and had inclusions that were comparable in size to the other transformants (Table 6). These observations could indicate that the fitness costs of transformation were plasmid- and strain-dependent.

In all of the transformation experiments, most of the inclusions grew slowly during passaging. Thus, only the healthier isolates were selected for further experiments. In one experiment shown in Figure 2, there were no significant differences between inclusions sizes of the complemented strains (*tc0439* with the native *tc0600* & *tc0439* with the empty vector) compared to the *tc0439* mutant and wt CM.

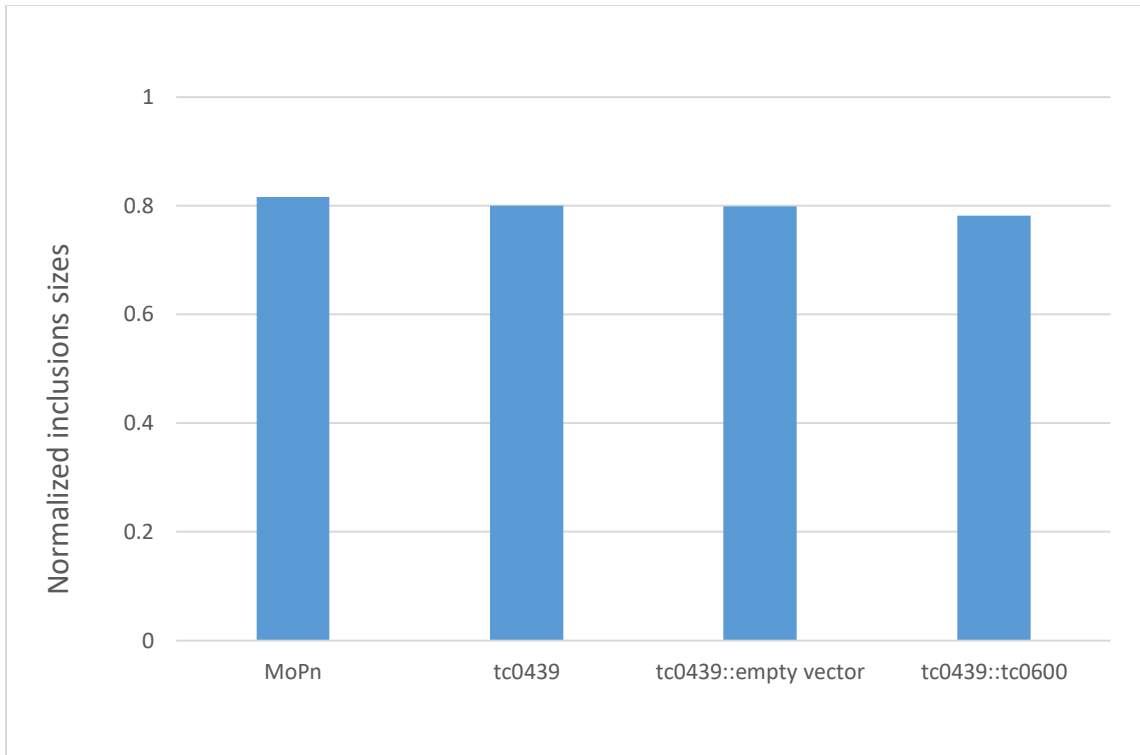


Figure 2. Normalized Inclusions Sizes of the Complemented Strains. Inclusion sizes were normalized by dividing the inclusions sizes in CMT-93 cells by the inclusions sizes in McCoy cells. The inclusion size of the complemented strains with native *tc0600* was slightly reduced compared to the inclusions of wt CM, the *tc0439* mutant, and the *tc0439* mutant transformed with the empty vector.

3.2 *tc0439* is Partially Attenuated *in vitro*

In both McCoy and CMT-93 cells, the inclusions ratio was obtained by dividing the average number of inclusions in the infected CMT-93 cell by the average number of inclusions in the mice GI epithelial cells, for each condition, in the presence or absence of cycloheximide, and the number of inclusions formed by the *tc0439* mutant was compared to wt CM (Figure 3). The *tc0439* mutant was partially attenuated in murine GIT cells.

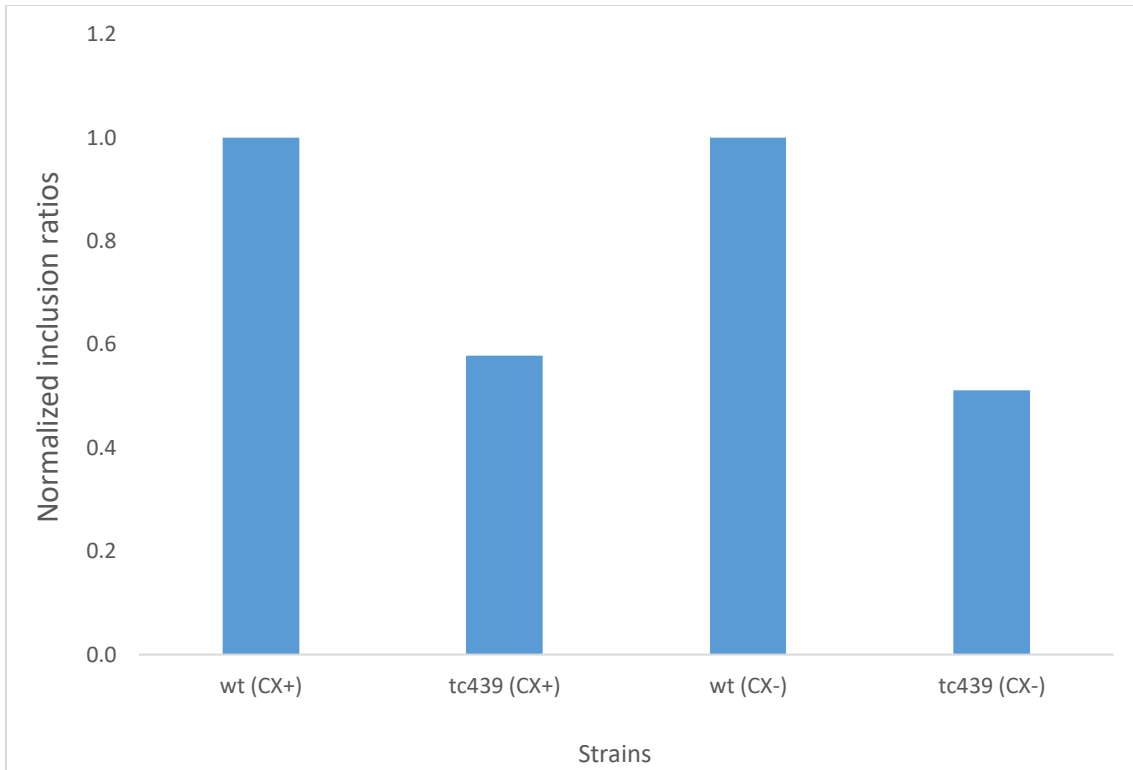


Figure 3. Inclusions Ratios of wt CM and the *tc0439* Mutant. Inclusion numbers were normalized by dividing the number of inclusions formed in CMT-93 by the number of inclusions formed in McCoy cells, for each strain, in the presence and absence of cycloheximide (CX+ and CX-). The *tc0439* mutant was partially attenuated both in the presence and i absence of cycloheximide compared to wt CM.

We used a similar methodology to compare the size of the inclusions the various strains formed in McCoy and CMT-93 cells (Figure 4). The *tc0439* mutant inclusions were similar in size to the inclusions of wt CM in both the presence and absence of cycloheximide.

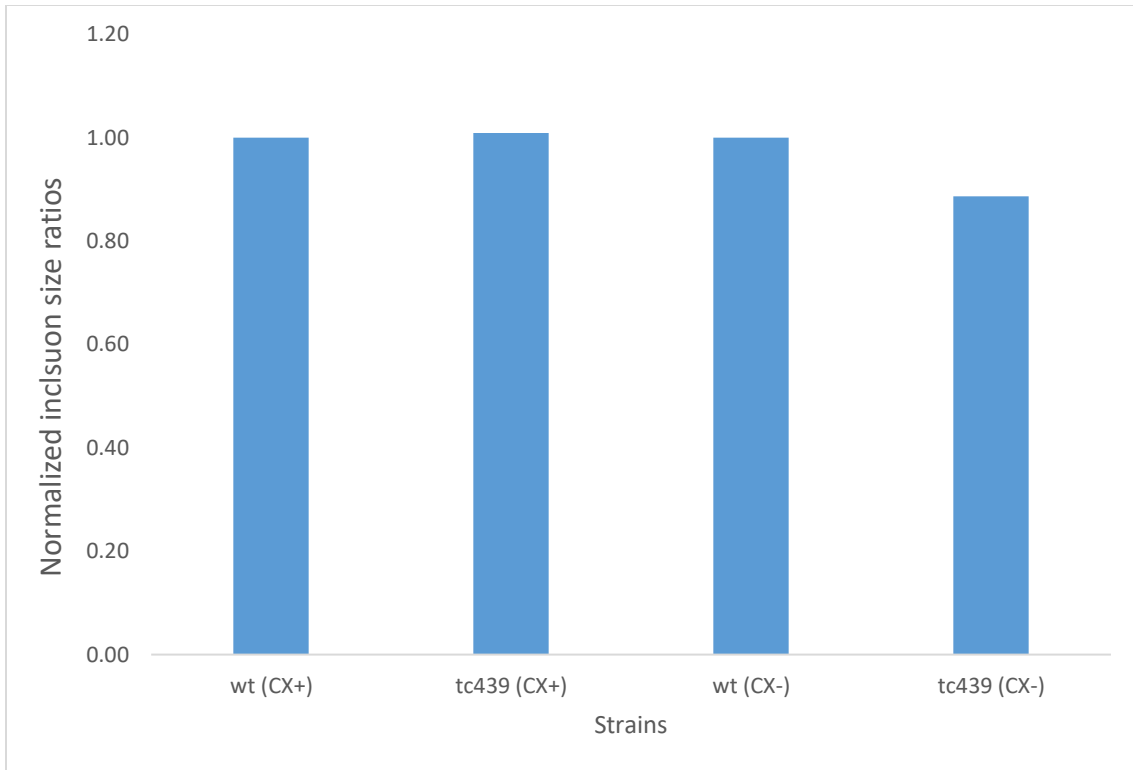
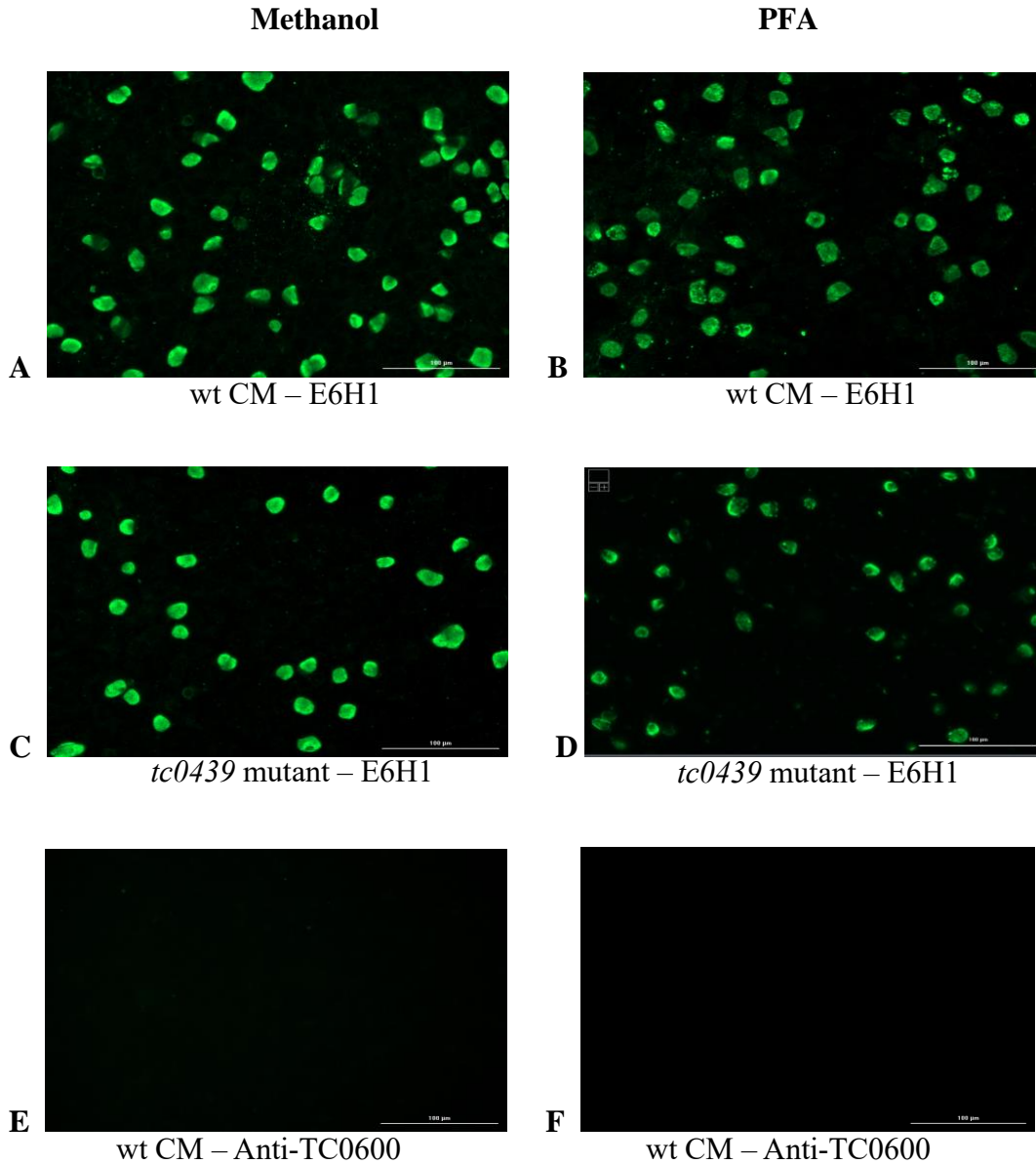


Figure 4. Size of wt CM and *tc0439* Mutant inclusions. Inclusions sizes were normalized by dividing the inclusion sizes of each strain in CMT-93 cells by their size in McCoy cells, in the presence or absence of cycloheximide. In the absence of the cycloheximide, the *tc0439* mutant formed slightly smaller inclusions, and *tc0439* mutant inclusions were smaller than wt CM inclusions in both conditions.

3.3 Failure to Detect Native *tc0600* and 1xFLAG-tagged *tc0600* by IFA

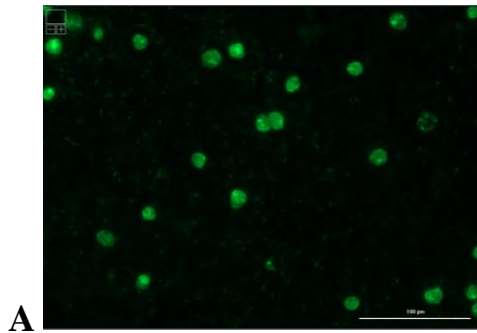
In all of the IFA experiments that I conducted in a 96-well plate format, the positive control (E6H1) worked as expected (Picture 2, Picture 3, and Picture 4). In contrast, no specific labeling was observed in any of the strains that I labeled with primary polyclonal Anti-TC0600, regardless of the incubation time, fixation, or infection conditions (E & F Picture 1).

Similar results were observed when I attempted to label TC0600 on coverslips in a 24-well plate format. In contrast, I observed robust labeling of chlamydial LPS using E6H1 in all of the conditions. Similarly, no positive labeling was observed when I stained the same strains with a primary monoclonal Anti-FLAG M2 (Sigma) antibody (B & C in Picture 2). However, I did observe labeling of 3X Flag-tagged IncV when I expressed it in the *tc0439* mutant from P2TK2 and labeled the infected cells with primary monoclonal Anti-FLAG M2 (Sigma) Ab and a secondary Anti-mouse Ab (image D in Picture 2 & Picture 3). Similar results were observed when I used an IFA labeling approach with paraformaldehyde fixation (Picture 3). Images in the left panel in Picture 2 and Picture 3 represent the positive control (E6H1) which also worked as expected in each strain and condition. Overall, we concluded that our polyclonal antibodies were unable to detect native or recombinant versions of Tc0600.



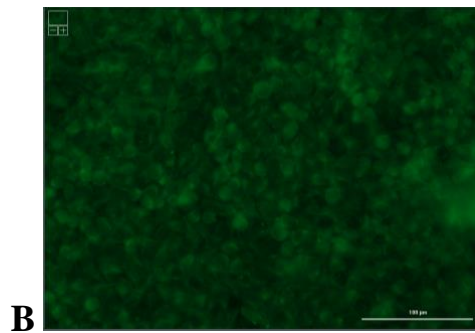
Picture 1. IFA Results for Native *tc0600* Fixed with Methanol or PFA. In E and F images, wt CM was labeled with primary antibodies (polyclonal Anti-TC0600) generated against *tc0600* and secondary FITC-conjugated anti-rabbit Abs. No positive labeling of Tc0600 was observed using either methanol or paraformaldehyde fixation. Positive controls are shown in wt CM and the *tc0439* mutant in images A thru D (primary E6H1 and secondary FITC-conjugated anti-mouse Abs).

1° Abs (E6H1)
2° Abs (Anti-mouse)

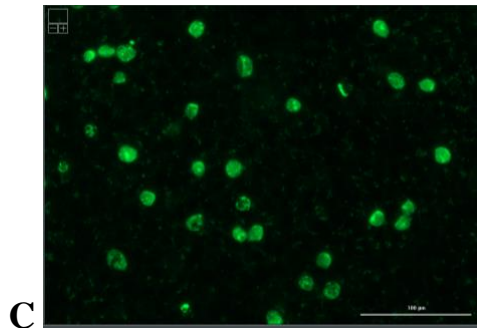


A TC0439::*tc0600*::1xFLAG

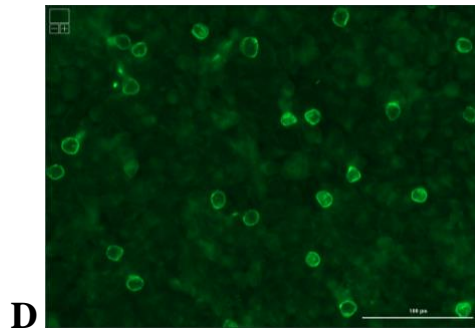
1° Abs (Anti-FLAG)
2° Abs (Anti-mouse)



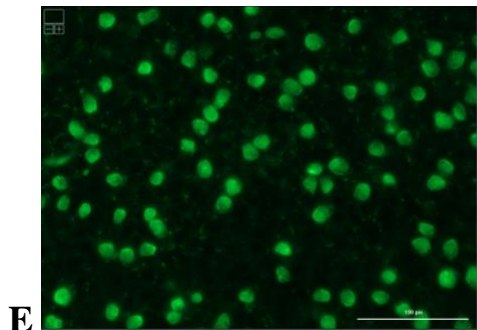
B TC0439::*tc0600*::1xFLAG



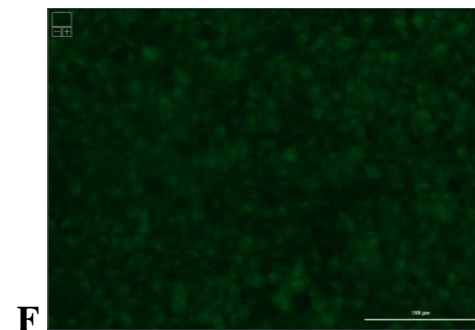
C TC0439::empty-vectorP2TK2



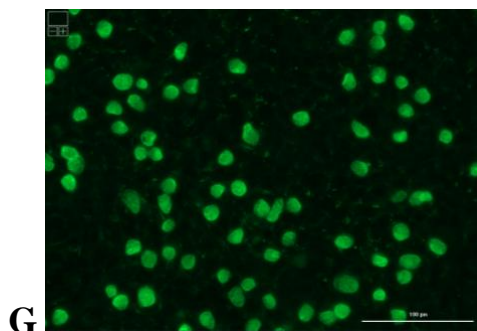
D TC0439::empty-vectorP2TK2



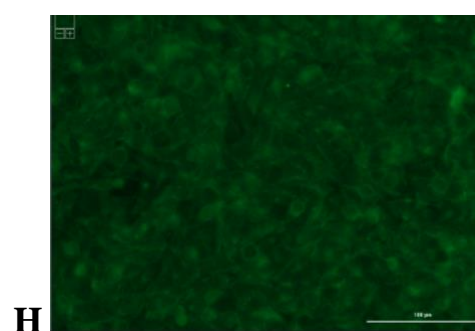
E *tc0439* mutant



F *tc0439* mutant



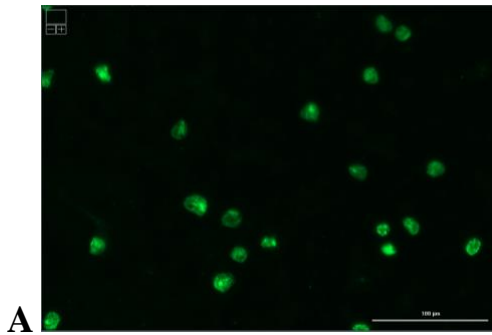
G wt CM



H wt CM

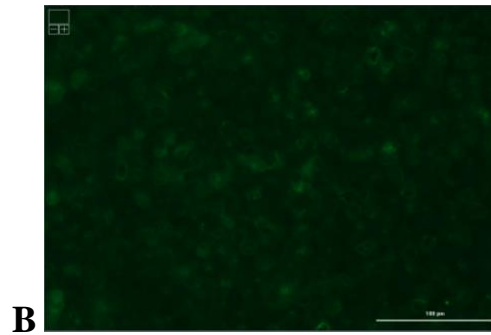
Picture 2. IFA Results for *tc0600::1xFLAG* Fixed with Methanol. This IFA experiment was conducted to detect the expression of FLAG-tagged *tc0600* in the *tc0439* mutant. All cells in these pictures were fixed with methanol. No positive signals were observed in the FLAG-tagged *tc0600* transformant (image B). The positive control for the Anti-FLAG Abs was the empty vector, PTTK2, which expresses the 3X FLAG-tagged chlamydial membrane protein (IncV) (images C and D). The positive control for the Anti-FLAG Abs yielded the expected ring shape fluorescence pattern indicating inclusion labeling (image D). wt CM and the *tc0439* mutant were included in this experiment as positive controls (images E, F, G, and H).

1° Abs (E6H1)
2° Abs (Anti-mouse)

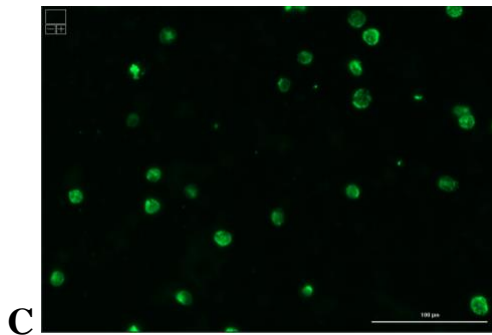


A TC0439::*tc0600*::1xFLAG

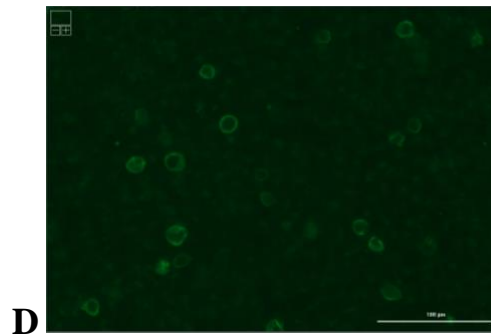
1° Abs (Anti-FLAG)
2° Abs (Anti-mouse)



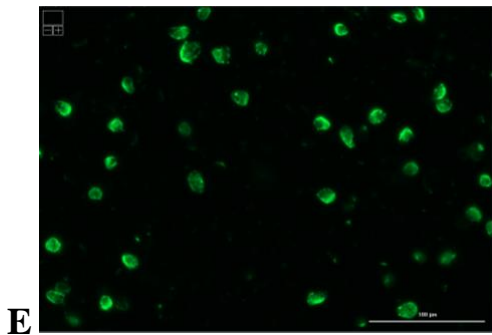
B TC0439::*tc0600*::1xFLAG



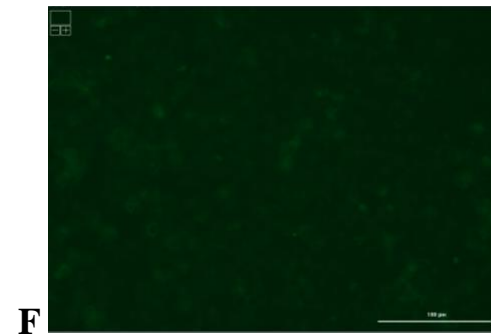
C TC0439::empty-vector



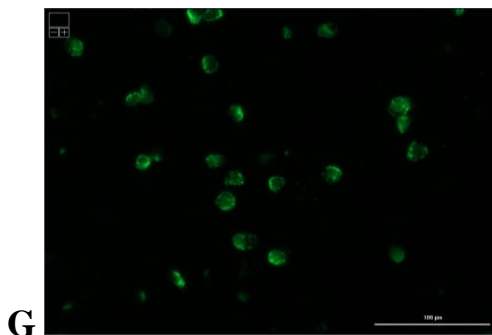
D TC0439::empty-vector P2TK2



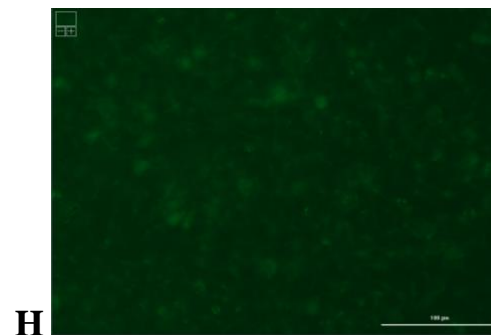
E *tc0439* mutant



F *tc0439* mutant



G wt CM



H wt CM

Picture 3. IFA Results for *tc0600::1xFLAG* Fixed with Paraformaldehyde.

This IFA experiment was conducted to test if I could detect expression of FLAG-tagged *tc0600* in the *tc0439* mutant. All of the cells were fixed with paraformaldehyde. No positive signals were observed when I labeled the FLAG-tagged *tc0600* transformant in image B. The positive control was the empty vector, PTTK2, in the *tc0439* mutant (images C and D). The positive control yielded a ring-shaped fluorescence staining of the inclusion, as expected (image D). wt CM and the *tc0439* mutant were included as a positive controls for IFA analysis (images E, F, G, and H).

3.4 Tc0600 Western Blot

I suspected that the epitope that the polyclonal anti-TC0600 antibodies were prepared against might not be exposed by the relatively gentle fixation conditions I used during IFA microscopy, so I tested if I could detect expression of TC0600 by using denaturing gel electrophoresis followed by Western blot. Strains used in the experiments included the *tc0439* mutant complemented with native TC0600, wt CM strains, and the *tc0439* mutant transformed with the Flag-tagged TC0600 construct. Although, I tested several conditions, such as different concentrations of antibodies and incubation times, no positive signals were observed in any of the strains expressing native or recombinant versions of TC0600. In contrast, I was able to detect labeling of the host protein GAPDH in these experiments, suggesting that my failure to detect expression of TC0600 could indicate that the antibodies could not recognize TC0600 or that this protein was not being expressed using the infection conditions that I selected.

Discussion

Multiple biovars of CT infect humans, and chlamydia is the most common bacterial STD. However, CT can infect the human GIT for extended periods without causing disease. Long-lasting colonization of the GIT by CT is also usually asymptomatic, although the similar invasive LGV biovar (serovar L1-L3) strains can cause proctitis. The colonization of the mouse GIT by CM can elicit a protective transmucosal immune response, which protects these mice against subsequent genital infection [54]. However, it remains unclear if CT infections of the GIT elicit similar transmucosal immune protection against genital tract chlamydia in humans.

We performed this study because we were interested in identifying genes that are required for chlamydial GIT tropism in mice and humans. Three mutagenized isolates of CM, *tc0437*, *tc038*, and *tc0439*, obtained by Rajaram et al. in 2015, displayed canonical pathology in mice genital tract. However, when the mice were infected via gavage and the rectum, the *tc0439* mutant was highly attenuated in mice GIT compared to WT CM and the other mutants. An additional nonsense mutation was detected in the *tc0439* mutant in *tc0600*. The CT ortholog of *tc0600*, *ct326*, is believed to encode an inclusion membrane protein [15]. This gene has also been linked to CT GIT tropism in humans [27]. Based on this observation, and since about 99% of CM genes have orthologs in CT 99%, we suspected that the mutation in *tc0600* might be driving the GIT attenuation of the *tc0439* mutant strain in mice.

We compared the ability of the *tc0439* mutant and the WT parent to form inclusions in two different cell lines, mouse fibroblasts (McCoy) and mouse epithelial cells (CMT-93). Based on the ratio of inclusions obtained in McCoy and CMT-93 cells,

tc0439 may only be subtly attenuated *in vitro*, either in the presence and absence of cycloheximide, compared to WT. This *in vitro* result did not reflect the strong *in vivo* phenotype observed by Morrison et al. (2018). This might be due to differences in the microenvironment of the mice GIT and our *in vitro* model, or interaction of the mutant with immune factors or cells that are not present in our *in vitro* model system.

Complementation of the *in vitro* phenotype of the *tc0439* mutant was the primary goal of my project. I successfully introduced native *tc0600*, as well as the corresponding empty cloning vector, into the *tc0439* mutant. In addition, I recently introduced a vector encoding a flag-tagged version of *tc600*. However, the transformed strains were not obtained at the same passage during the transformation experiments. This could indicate that these constructs alter chlamydial fitness, and possibly, that over-expression of TC0600 and variants of this protein is detrimental.

Results of my attempts to detect TC0600 expression and localization using polyclonal Anti-TC0600 Abs and Anti-FLAG M2 (Sigma) antibody, were unsuccessful. This could indicate that low levels of these proteins were expressed, or that my labeling methods need to be optimized. An immediate future goal of my project is to attempt localization using a 3X-flagged version of TC0600 because this strategy has worked well for the localization of other chlamydial proteins.

During our attempt to produce polyclonal Anti-TC0600 Abs (Sino Biological), it was difficult to find antigenic domains in the TC0600 peptide sequence. The antigenic regions that were predicted and selected had three cysteine residues, which could interfere with conjugation (Table 4). Thus, two of three cysteine molecules were replaced with serines, and the mutant peptide was used to immunize rabbits. This could have

resulted in polyclonal Anti-TC0600 with low affinity for the target protein. In the context of immunofluorescence assays, it is also possible that the epitope against which the polyclonal antibody was raised is not surface exposed. One possible future direction to mitigate this would be to use more stringent fixation and permeabilization protocols to ensure that TC0600 is maximally unfolded.

In vivo, the *tc0439* mutant was highly attenuated in mice GIT compared to WT [34]. However, our investigation of the phenotype of this mutant *in vitro* revealed only subtle growth defects. This could indicate that we did not use the proper cell types to tease out a phenotype; it will be important to evaluate if this mutant is attenuated in other cell types, like macrophages and neutrophils, that CM might encounter in the murine GIT.

A critical future direction is to test if the virulence of the *tc0439* mutant is rescued in the mouse GIT when we add back TC0600. We will infect mice with the complemented strain (TC0439::*tc0600*), the strain transformed with the empty vector (TC0439::P2TK2), and the WT parent transformed with the empty vector (WT CM::P2TK2) rectally or via oral gavage, to differentiate potential confounding effects of expressing TC0600 from a shuttle vector. If TC0600 is responsible for the phenotype of the mutant, we expect that mice infected with the complemented strain will shed comparable levels of EBs to the wild type parent strain, whereas the strains carrying the empty vector would phenocopy the mutant.

As mentioned previously, the *tc0439* mutant has two nonsense mutations, one in *tc0439* and one in *tc0600*. Some mutants in our collection contain sub-sets of the mutations present in different strains. For instance, the *tc0437/tc0439* mutant contains the

nonsense mutations in *tc0437* and *tc0439* genes, the latter of which is similar to the mutation in *tc0439* in the *tc0439* mutant. However, the *tc0437/tc0439* mutant is not attenuated in the mouse GIT [34]. This provided some of the original evidence that the nonsense mutation in *tc0439* of the corresponding strain does not mediate the GIT phenotype. However, another mutant, *guaB*, shares 7 mutations with *tc0439* mutant. One of mutation common to both of these mutants is the nonsense mutation in *tc0600*. Notably, the *guaB* mutant displayed similar phenotypes to the *tc0439* mutant in murine GIT and genital models [34]. This is the best direct evidence that *tc0600* could mediate the tropism phenotype, but it is possible that other mutations in other genes mediate GIT attenuation. One of our future goals, besides studying the *tc0439* mutant, is to test of introduction of native TC0600 and other proteins that are mutated in the *tc0439* and the *guaB* mutants restore virulence of these mutants in the GIT. In doing so, we may be able to identify chromosomal factors that mediate chlamydial gastrointestinal tropism, which would be a novel and paradigm-shifting observation.

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