Chlamydia muridarum Genital and Gastrointestinal Infection Tropism is Mediated by Distinct Chromosomal Factors

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Running Head: Tissue Specific Chlamydia Mutant

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Some members of the genus *Chlamydia*, including the human pathogen *Chlamydia trachomatis*, infect multiple tissues including the genital and gastrointestinal (GI) tracts. However, it is unknown if bacterial targeting to these sites is mediated by multifunctional or distinct chlamydial factors. We previously showed that disruption of individual large clostridial toxin homologs encoded within the *Chlamydia muridarum* plasticity zone were not critical for murine genital tract infection. Here, we assessed if cytotoxin genes contribute to *C. muridarum* GI tropism. Infectivity and shedding of wild-type (WT) *C. muridarum* and three mutants containing nonsense mutations in different cytotoxin genes, tc0437, tc0438, and tc0439, were compared in mouse genital and GI infection models. One mutant, that had a nonsense mutation in tc0439, was highly attenuated for GI infection and had a GI ID$_{50}$ that was 1000 times greater than WT. GI inoculation with this mutant failed to elicit anti-chlamydial antibodies or to protect against subsequent genital tract infection. Genome sequencing of the tc0439 mutant revealed additional chromosomal mutations, and phenotyping of additional mutants suggested that the GI attenuation might be linked to a nonsense mutation in tc0600. The molecular mechanism underlying this dramatic difference in tissue-tropic virulence is not fully understood. However, isolation of these mutants demonstrates that distinct chlamydial chromosomal factors mediate chlamydial tissue tropism, and provides a basis for vaccine initiatives to isolate chlamydia strains that are attenuated for genital infection, but retain the ability to colonize the GI tract and elicit protective immune responses.
INTRODUCTION

*Chlamydia trachomatis* is the most common bacterial sexually transmitted infection in the US, with nearly 1.6 million cases reported to the US CDC in 2016 (1). Many *C. trachomatis* infections are asymptomatic so the actual number of cases is likely to be much higher (2-4). When chlamydia is diagnosed, it is effectively treated with antibiotics, however, undetected genital infections in women can persist and ascend, to cause infection and subsequent inflammation in the upper genital tract (5). Upper genital tract inflammation may cause pelvic inflammatory disease, salpingitis, tubal scarring, ectopic pregnancy and infertility. In addition to the urogenital tract, *C. trachomatis* infects the conjunctiva, pharynx, respiratory tract, rectum and the gastrointestinal (GI) tract. The recent recognition that rectal infections are frequent in women who do not report traditional rectal chlamydia risk factors (6), and the premise that the GI tract may serve as a site for persisting infection and as a reservoir for urogenital re-infection (7-10), has renewed interest in understanding the molecular basis of chlamydial GI colonization and pathogenesis.

The chlamydial plasticity zone (PZ) is a region of high genetic diversity in otherwise highly conserved *Chlamydia* spp. genomes (11, 12). Some PZ genes mediate chlamydial immune evasion, but the functions of most of these genes are unknown (13-16). Using a murine genital tract infection model, we previously assessed the virulence of *C. muridarum* mutants containing nonsense mutations in various PZ genes, including three cytotoxin genes (*tc0437, tc0438* and *tc0439*) (17). These mutants elicited genital infections similar to their parent as assessed by bacterial shedding and duration of infection, although infection with the mutants generally resulted in less hydrosalpinx (17). However, because the chlamydial cytotoxin genes share homology with large clostridial toxin genes (18), which are key *Clostridium difficile* GI virulence factors (19), we sought to determine if the *C. muridarum* cytotoxin mutants showed altered virulence for GI infection.
In the current study, the pathogenicity of the three cytotoxin nonsense mutants was compared in two GI infection models by assessing bacterial shedding, anti-chlamydia antibody responses, and immunity to genital infection. We found that nonsense mutations in individual cytotoxin genes per se did not alter the virulence of *C. muridarum* infection for either genital or GI infections. However, the profound attenuation of one of the cytotoxin mutants for GI, but not genital, infection was associated with an additional background nonsense mutation in the conserved chlamydial hypothetical protein gene *tc0600*, suggesting that this gene might be a GI-specific virulence factor. Overall, our results show that distinct chromosomal genes mediate *C. muridarum* genital and GI infection and suggest that it might be possible to construct vaccine strains that are attenuated for genital tract infection, but are able to colonize the GI tract to elicit transmucosal genital tract protection.

RESULTS

*C. muridarum* cytotoxin mutants are virulent in the murine genital tract. The goal of the current study was to determine if *C. muridarum* cytotoxin mutants have tissue-specific virulence defects. Therefore, it was first important to verify the virulence of all strains in the genital infection model (17) and to measure baseline immune responses for comparison to GI infections. Mice were intravaginally inoculated, with the wild-type parent strain (WT) or with mutants (TC0437, TC0438 or TC0439) having nonsense mutations in one of the three cytotoxin genes (*tc0437, tc0438* or *tc0439*, respectively), and infection was monitored by enumerating chlamydiae collected from cervico-vaginal swabs. The magnitude and duration of chlamydiae shedding between WT and mutant inoculated animals was similar, with the only significant difference detected between WT and TC0438 at day 10 (p<0.016) (Fig. 1A). Furthermore, the overall immunoglobulin class and subclass specific anti-chlamydia antibody responses elicited by infection with the mutants was not markedly different from WT, but slight differences were observed in titers of some responses. (Fig. 1B). Thus, measures of chlamydial
shedding, infection duration, and immunogenicity, confirmed that disruption of individual cytotoxin genes did not overtly impact the virulence of *C. muridarum* in the mouse genital tract.

*C. muridarum* mutant TC0439 displays unique tissue–specific virulence. Chlamydiae have been isolated from the GI tract of both animals and humans (7). In mice, *C. muridarum* establishes long-lasting GI infections characterized by minimal tissue pathology (10, 20-23). Because *C. muridarum* cytotoxin genes are homologous to genes that are critical for colonization of the GI tract by other bacterial pathogens (24, 25), we sought to determine if the GI tropism of chlamydial cytotoxin mutants is altered. GI infections were established by intrarectal (Fig. 2A) or oral gavage (Fig. 2B) inoculation. WT produced long-lasting infections by both inoculation routes. GI shedding from WT infected mice measured between $10^3$ and $10^4$ inclusion forming units (IFUs) during early infection (1 to 4 wk), then decreased to $10^1$ to $10^3$ IFUs later in infection (5 to 11 wk). The course of GI infections produced by either inoculation route with TC0437 and TC0438 were remarkably similar to WT, although mice inoculated intrarectally with TC0438 did resolve GI infection by 8 wk post inoculation (p<0.008). In contrast, the attenuation of TC0439 in both GI infection models was striking (Fig. 2). Neither intrarectal nor oral gavage inoculation with TC0439 resulted in productive infection (p<0.001). Thus, TC0439 displayed a unique tissue-restricted virulence phenotype, retaining full virulence for genital tract infection (Fig. 1), while exhibiting highly-attenuated GI infection (Fig. 2).

**Immunological studies corroborate the attenuation of TC0439 for GI infection.** We inferred, from the chlamydial shedding data collected by rectal swabs, that TC0439 was attenuated for GI infection. An alternative explanation for that observation is that TC0439 established infection in the upper GI tract or disseminated to distant tissues, and rectal culture failed to detect those infections. We differentiated those possibilities by measuring the anti-chlamydial antibody responses and the ability of GI-challenged mice to defend against genital reinfection. Infection of the murine GI tract with WT by intrarectal (Fig. 3A) or oral gavage (Fig. 3B) inoculation, elicited robust anti-chlamydia antibody responses. Similar
antibody responses developed following infection with TC0437 and TC0438. In contrast, the antibody responses elicited following intrarectal (Fig. 3A) or oral gavage (Fig. 3B) inoculation with TC0439 were below or slightly above baseline levels of uninfected mice (p<0.02).

The inability of TC0439 to productively infect the GI tract and induce anti-chlamydial antibodies suggested that its GI virulence was severely impaired. As an independent virulence measure, we assessed whether GI infection with TC0439 protected against subsequent genital tract challenge (23).

Mice were challenged by either intrarectal or oral gavage inoculation with WT or TC0439, and GI infection was monitored by rectal swab for 11 weeks (Fig. 4A). Approximately 17 weeks post-inoculation, mice were treated with doxycycline to cure residual infection, treated with Depo-provera and challenged vaginally with the corresponding chlamydial strain. Mice infected with WT, by either intrarectal or gavage inoculation, were markedly protected against WT genital tract infection (p<0.0005) (Fig. 4B). In contrast, GI inoculation of mice with TC0439 did not protect against subsequent TC0439 genital infection. The course of genital infection (chlamydiae shedding and infection duration) in mice receiving a prior GI challenge with TC0439 was remarkably similar to primary genital tract infection with this mutant (Fig. 1A). Moreover, the marginal anti-chlamydia antibody response elicited by TC0439 following GI infection, was increased to a level typically observed following primary vaginal challenge (Fig. 5A and 5B). Also, 4 days prior to vaginal challenge (143 days post-GI infection), all GI WT-challenged mice (5/5 rectal infected and 4/4 gavage infected) were vaginal wash positive for anti-chlamydia antibody, whereas 0/5 rectal and 1/5 gavage TC0439 challenged mice were vaginal wash positive for anti-chlamydia antibody. Collectively, these results confirmed that TC0439 was highly attenuated for GI infection.

**GI attenuation of TC0439 is independent of challenge dose.** GI infection established by either oral gavage or intrarectal inoculation of WT resulted in similar infection courses, as based upon rectal bacterial shedding and serology (Figs. 2 and 3). Therefore, we used the intrarectal inoculation model to...
compare the infectious dose response of WT to TC0439 (Fig. 6). For the purpose of calculating an infectious dose 50% (ID$_{50}$), we evaluated infection at 21-days post-challenge. Based upon our previous experiments (Fig. 2), 21-days post-challenge was sufficient to establish, but not resolve, infection. The dose response to WT infection was assessed by challenging mice by intrarectal inoculation with either $10^2$, $10^3$, $10^4$, or $10^5$ IFUs. All mice challenged with $10^5$ or $10^4$ IFUs became infected, whereas intrarectal challenge with $10^3$ or $10^2$ IFUs resulted in Gi infection of 3/6 and 0/6 mice, respectively. The estimated ID$_{50}$ for WT Gi infection by intrarectal inoculation was $10^{2.97}$ IFUs (95% CI $10^{2.23}$, $10^{3.65}$). Challenge doses of $10^3$, $10^4$, $10^5$, $10^6$, and $10^7$ IFUs were used to assess the dose response of TC0439. None of the doses were sufficient to infect 100% of the mice. Five of 6 mice inoculated with $10^8$ IFUs were infected, but the shedding of chlamydiae from these animals was much lower than animals infected with WT. The estimated ID$_{50}$ for TC0439 Gi infection by intrarectal inoculation was $10^{6.85}$ IFUs (95% CI $10^{6.18}$, $10^{7.59}$), which was significantly different from WT ($p<0.0003$). TC0439 was not only more than a 1000-fold less infectious than WT for Gi infection, mice that did become culture positive following challenge with high doses of the mutant shed > 100-fold fewer chlamydiae than mice infected with WT. Ninety-four days following challenge, sera were analyzed for anti-chlamydial antibody. All culture positive (infected) mice that had been inoculated with either WT or TC0439 were seropositive whereas none of the culture negative mice seroconverted, thus verifying the infectivity data.

**Background chromosomal nonsense mutation might explain profound Gi attenuation.** TC0439 was generated by four sequential rounds of chemical mutagenesis and contains 28 point mutations in comparison to WT, including nonsense mutations in tc0439 and the conserved chlamydial ORF tc0600 (Table 1). To determine if Gi attenuation of TC0439 might be linked to inactivation of tc0439, tc0600, or other missense mutations, we evaluated the phenotypes of two additional mutants, TC0437/0439 and GuaB (Table 1, Fig. 8), in the genital tract and Gi infection models. TC0437/0439, contains nonsense mutations in tc0437 and tc0439, but encodes tc0600, while GuaB contains nonsense mutations in guaB.
and tc0600, but encodes tc0439. Genital infection resulting from vaginal challenge with TC0437/0439 and GuaB resembled WT infection (Fig 7A), although chlamydial shedding was somewhat reduced compared to WT (p<0.05). However, when the mutants were compared to WT in the rectal infection model, TC0437/0439 behaved similar to WT, though shedding was somewhat reduced (p<0.05), whereas mutant GuaB was strikingly attenuated (p<0.0001), with few animals displaying evidence of productive infection (Fig. 7B). The marked attenuation of TC0439 and GuaB for GI infection provides compelling evidence that distinct chromosomal genes strongly impact the tissue-specific virulence of Chlamydia. Furthermore, a shared mutation in the genomes of TC0439 and GuaB (Table 2 and Fig. 8) resulted in dramatic GI attenuation.

DISCUSSION

Chlamydia spp. colonize multiple distinct organ system tissues (7). In humans, C. trachomatis strains most frequently infect the cervix and urethra, but can also colonize the pharynx, conjunctiva and GI tract. Chlamydial GI infections are often asymptomatic, can persist without overt signs or symptoms of infection, and are less sensitive to the standard of care antibiotic therapy (single dose azithromycin) (6, 8, 9, 26-28). Because of the propensity of GI infections to persist, even following antibiotic therapy, it has been proposed that chlamydial GI infection is a reservoir for genital tract reinfections (7-10). The recent realization that chlamydia GI infections are common in women has renewed interest in understanding the pathogenesis and the pathological consequences of GI chlamydial infection, and elucidating the role of these infections in the epidemiology of the more problematic female urogenital infections.

The murine model using C. muridarum is an ideal experimental system to study tissue-specific virulence factors of Chlamydia. C. muridarum colonizes both the genital and GI tracts, and produces distinct tissue-specific experimental outcomes that mimic many characteristics of human genital and GI
infections. GI colonization with *C. muridarum* can be established by oral or rectal inoculation, or as the result of dissemination via the blood from a genital infection (29). GI infection often persists and is characterized by long-term shedding of infectious chlamydiae in the absence of overt inflammation (10). Conversely, genital infection is characterized by the shedding of abundant infectious chlamydiae and significant inflammation, and infection naturally resolves in about 4 weeks (30, 31).

A few studies have used the murine infection model to study chlamydiaal tissue tropism (32-34). In one recent study, the *C. muridarum* plasmid gene, pgp3, was associated with GI but not genital infection (34). However, our current study clearly demonstrates tissue-specific virulence factors are also encoded on the *C. muridarum* chromosome. We speculate that a nonsense mutation in the chromosomally encoded gene tc0600 was independently associated with GI-specific attenuation, but further genetic studies are needed to precisely map the chromosomal gene(s) responsible for the GI-specific attenuation. Interestingly, polymorphisms in the tc0600 *C. trachomatis* ortholog ct326, which encodes a putative secreted inclusion membrane protein (35), have been associated with GI tropism in humans. A 111 nucleotide in-frame insertion in ct326 of *C. trachomatis* serovar G is correlated with the ability of this strain to infect the GI tract, and strains lacking this insertion are associated with cervical, but not GI infection (36). This 111 nucleotide region is conserved in *C. muridarum* tc0600.

Although we associated inactivation of tc0600 with GI attenuation, the TC0439 and GuaB mutant strains share seven additional non-synonymous mutations (Table 2 and Fig. 8). The shared mutations suggest that these isolates were derived from a common parent that was present in the population after the first few rounds of mutagenesis. Four of these mutations are predicted to cause non-conserved amino acid changes in: TC0054 (penicillin binding protein), TC0290 (hypothetical), TC0312 (glycosyl hydrolase, glgx), and TC0473 (peptide ABC transporter, permease protein). These genes have not been linked to *C. muridarum* virulence or reported to be plasmid-regulated (37-39).

Identifying the specific allele(s) responsible for attenuation of the TC0439 and GuaB mutant strains will
be essential to understanding the mechanism of GI attenuation. Unfortunately, complementation is contraindicated for differentiating the potential mutant alleles, because the plasmid is a *C. muridarum* GI virulence factor (32) and negative complementation results could indicate failure to complement or cis-acting effects on the plasmid. Perhaps one method that could be used to circumvent this concern, would be to use the FRAEMING approach developed in *C. trachomatis* to inactivate *tc0600*, which would permit generation of targeted chromosomal deletions while maintaining the endogenous plasmid (40).

Also of interest is the nonsynonymous SNP in *tc0439* contained in the GuaB mutant (aa residue 3114 of the TC0439 mutant), which results in a glycine to aspartic acid change. Sequence comparison of cytotoxin genes from a variety of pathogens shows that this glycine residue is highly conserved, and thus mutating such a highly conserved residue could result in the loss of toxin function. Because this *tc0439* mutation is found in the GuaB mutant, and the TC0439 mutant contains a premature stop codon in *tc0439*, perhaps the attenuated GI infection phenotype observed with these two mutants was due to loss of function of the *tc0439* toxin. However, the TC0437/0439 mutant, which contains a premature stop codon in *tc0439* upstream of the nonsynonymous SNP in *tc0439* contained in the GuaB mutant, remains infectious for the GI tract and thus does not corroborate that notion. The multiple mutations present in our strains confounds the identification of a specific gene mutation responsible for the tissue-tropic phenotype with absolute certainty, and the infection phenotype that we report could be multifactorial. Nevertheless, the very robust in vivo infection phenotype provides a compelling basis for further studies to elucidate the molecular mechanism of tissue-specific infection.

Short-lived protective immunity develops following human *C. trachomatis* genital infection (41-43), whereas in mice, protective immunity is quite long-lasting (30, 44, 45). This dissimilarity in the duration of immunity between humans and mice is not understood and could be multifactorial. However, our observation and those of others showing that murine GI *C. muridarum* infection produces nearly sterilizing immunity against vaginal challenge (Fig.4) (23) may be central to elucidating the
immunological mechanisms responsible for the durability of chlamydial immunity. Mice infected
vaginally with *C. muridarum*, concurrently acquire GI infection, and remain GI tract positive long after
genital infection has resolved (46). Perhaps, then, persisting GI infection is a vital component for the
robust durability of protective immunity, and could be key to developing an efficacious vaccine to
prevent chlamydial genital infection. We clearly showed that genital and GI infection tropisms were
separable by identifying a mutant that infected the genital tract normally, but was highly attenuated for
GI infection. While that is not the phenotype desired for a chlamydial vaccine, it does provide proof-of-
principle for the identification of strains that possess altered tissue tropism. Identification of strains
that infect the GI tract, but are attenuated for genital tract infection, would allow for direct testing of
the hypothesis.

By using animal models of infection, a more complete understanding of the contribution of
chlamydial GI infection to the robust and long lasting protective immunity that develops against genital
infection will emerge. However, well-designed epidemiological studies and correlative immunological
analysis of subjects with chlamydial urogenital and GI infections will be needed to determine if a link
exists between GI infection and protective immunity to genital chlamydia in humans.

**MATERIALS AND METHODS**

*Chlamydia strains.* *C. muridarum* wild-type (WT) (GenBank accession AE002160.2) and *C.
muridarum* mutants, TC0437, TC0438, TC0439, GuaB (17), and TC0437/0439 (Table S1) were propagated
in HeLa 229 (47) cells and elementary bodies (EBs) were purified by discontinuous Renografin gradient
centrifugation. Table 1 summarizes the single nucleotide polymorphisms (SNPs) found in the mutants
(17) (Table S1).

*Generation and whole genome sequencing of a* *C. muridarum* TC0437/0439.* TC0437 (Table 1) was
mutagenized with 8 mg/mL ethyl methanesulfonate (EMS) and a clone that contained a nonsense
mutation in tc0439 (TC0437/0439) was identified, purified, and sequenced as described (17). Single nucleotide polymorphisms (SNP) and nucleotide insertions and deletions (indel) were mapped by aligning the TC0437/0439 and the C. muridarum reference genomes (Genbank accession number AE002160.2) using Bowtie2. SNPs/Indels were called using a Samtools mpileup function and remaining ambiguous sequences with low-quality scores were resolved by Sanger sequencing.

Mice. Female C57BL/6 mice, 6-to-8 weeks old, were purchased from Jackson Laboratories (Bar Harbor, ME), and maintained in the animal facilities at the University of Arkansas for Medical Sciences (Little Rock, AR). All experimental procedures were performed in accordance with protocols approved by the UAMS Institutional Animal Care and Use Committee.

Genital Infection. Five days before genital infection, mice were injected subcutaneously with 2.5mgs of medroxyprogesterone acetate (Depo-provera) (Greenstone, LLC) to synchronize the estrous cycle of experimental mice. Mice were then infected vaginally with 5 x 10⁴ IFUs of WT or mutants (48, 49). Infections were monitored by inoculating cervico-vaginal swab samples onto HeLa 229 cell monolayers and IFUs were visualized and counted as described in detail previously (48, 49).

Gastrointestinal (GI) Infection. Two routes of inoculation, intrarectal and gavage, were used to assess the pathogenicity of the mutants for the murine gastrointestinal tract. For rectal inoculation, the tip of a micropipette was gently inserted approximately 5 mm into the rectum and 10 μl of a buffered suspension [250 mM sucrose, 10 mM sodium phosphate, and 5 mM L-glutamic acid, pH7.2] (SPG) containing 1 x 10⁷ IFUs/ml of WT or mutants (1 x 10⁵ IFU total challenge dose) was deposited. Infection was monitored by collecting rectal swabs at weekly intervals. Calgiswabs (Puritan) were wet with SPG, inserted into the rectum, and rotated 8 times clockwise and 8 times counterclockwise. Swabs were then placed in a tube containing 0.5 ml SPG and two 4mm glass beads and vortexed at 1400 rpm for 3 minutes. The swabs were removed, an additional 0.5 ml of SPG was added and samples were stored at -80⁰ C. Infection was monitored by counting IFUs as described previously (48).
A flexible 20 gauge feeding tube (Instech) was used to challenge mice by gavage. Mice were inoculated with $1 \times 10^6$ IFUs of WT or mutants by depositing 100 μl of a suspension of $1 \times 10^7$ IFUs/ml in SPG into the stomach. Gastrointestinal infection was monitored by rectal swabs and IFUs were counted as described above (48).

**Vaginal challenge of GI infected mice.** Mice were infected with either WT or TC0439 by rectal or gavage inoculation, and infection was monitored weekly. One hundred and eighteen days following GI infection, mice received daily intraperitoneal injections of 300 μg of doxycycline for 10 days, which is sufficient to cure GI infection (9). Mice were then rested for 2 weeks to eliminate any residual effect of the antibiotic treatment and rectal swabs confirmed that mice were culture negative for chlamydiae.

Mice were then treated with Depo-provera and challenged vaginally with the homologous strain (WT or TC0439) as described above for genital infection.

**ELISA.** Anti-chlamydial antibody was measured by enzyme-linked immunosorbent assay (ELISA) using formalin-fixed density gradient purified WT EBs as antigen (30, 49).

**Statistical analyses.** Analyses of bacterial shedding data were performed using nonparametric longitudinal analysis methods (50). The methods are rank-based and require fewer distributional assumptions compared to parametric methods. The models included terms representing group (chlamydial strain), time (day of measurement) and a group-by-time interaction. As with other factorial models, the interaction effect is evaluated first and, if not significant, the overall group and time effects can be assessed. However, in the case of a significant interaction, the time profiles of each mutant were compared to WT, separately. Mutants whose time profile differed from WT were examined further by comparing the mutant to WT at each time point. Wilcoxon rank sum tests were used for these analyses. Wilcoxon rank sum tests were also used to compare mutants to WT with respect to antibody titer data. Finally, probit regression models were used to obtain ID$_{50}$ estimates and corresponding 95% confidence.
limits. P-values less than 0.05 were considered to be statistically significant. No adjustment for multiple comparisons were made.
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Figure 1. *C. muridarum* toxin mutants are virulent in the genital tract. C57BL/6 mice were pretreated with Depo-Provera and challenged with $5 \times 10^4$ IFUs of WT (n=6) or toxin mutants (n=6 for each mutant). (A) Vaginal vaults were swabbed at the indicated times and IFUs were enumerated on HeLa cell monolayers by immunofluorescence. Data are presented as geometric mean ± SD of IFUs recovered at the indicated days post-infection. Statistical analysis revealed differences in bacterial shedding over time among the strains (group x day p-value: 0.0049). Comparison of the WT profile to each of the mutant profiles revealed that WT was statistically different from TC0437 and TC0438, but not TC0439. TC0437 compared to WT approached statistically significant differences at day10 (p < 0.065) and Day 14 (p < 0.094), and TC0438 was significantly different from WT at day 10 (p < 0.016). No other differences were detected. (B) Sera collected 49 days following genital infection were analyzed by EB ELISA. Antibody responses are presented as mean log$_2$ titers ± SD for each of the indicated Ig classes. Dashed line indicates cut-off values for positive serologic response. The antibody responses were remarkably similar with the following exceptions: WT vs TC0437 and WT vs TC0439 IgA titers, p < 0.01; and WT vs TC0437 and WT vs TC0438 IgG3 titers, p < 0.05. No other differences were detected.

Figure 2. TC0439 is highly attenuated for GI infection. C57BL/6 mice were challenged by rectal (A) or gavage (B) inoculation with WT or mutants to measure virulence for GI infection. For rectal inoculation, 10µl containing $1 \times 10^5$ IFUs were placed intrarectally. Gavage inoculation was accomplished by inoculation of 100µl containing $1 \times 10^6$ IFUs using a feeding tube. Infection was assessed weekly by swabbing the rectum and counting IFUs on HeLa cell monolayers. Data are presented as geometric mean ± SD of IFUs at the indicated time points. Number of mice/group is indicated in the figure. For comparison, the infection curve for WT rectal (A) and gavage (B) infection is replicated in each panel. (A) Rectal challenge: The test of the overall interaction effect was found to be statistically significant (p < 0.0001). The interaction between TC0437 and WT was not significantly different (p > 0.63), and the
test of the main strain effect was not statistically significant (p > 0.063). The interaction effect for TCO438 and WT was statistically significant (p < 0.0001). Comparison performed for each day found significant differences from WT at day 56 and beyond (p < 0.008). Other time points were not significant. TCO439 was significantly different from WT at all time points (p < 0.001). (B) Gavage challenge: TCO437 was not found to be significantly different from WT (interaction: p > 0.085, main: p > 0.11); TCO438 was not found to be significantly different from WT (interaction: p > 0.19, main: p > 0.35); and TCO439 was found to be significantly different from WT (interaction: p < 0.020, main: p < 0.0001).

Figure 3. TCO439 fails to elicit robust antibody responses following GI infection. As an additional assessment of infection, the antibody responses of GI infected mice (Figure 2) were measured. Sera collected 81 days following rectal (A) or gavage (B) challenge was analyzed by EB ELISA. The number of mice per group is indicated on the figure. Antibody responses are presented as mean log_{2} titers ± SD for each of the indicated Ig classes. Dashed line indicates cut-off values for positive serologic response. The antibody responses of mice infected following rectal or gavage inoculation with mutant TCO439 were negative or just above background responses, and were significantly lower than responses elicited by WT infection (p<0.016 for all Ig classes tested, except IgG3 which was not significant, p>0.05). Antibody responses elicited by TCO437 and TCO438 were not significantly different from WT (p>0.05), except for TCO438 rectal challenge (IgG2b, IgG2c and IgA, p<0.008).

Figure 4. GI infection with TCO439 does not protect against genital challenge. Mice were infected by either gavage or intrarectal inoculation with WT or TCO439, and GI infection was monitored by enumerating IFUs from rectal swabs (A). At 118 days following GI infection, mice were treated with doxycycline for 10 days, rested 2 weeks, treated with Depo-Provera and then rechallenged vaginally with the homologous strain (B). Data are presented as geometric mean ± SD. The number of mice per group are indicated on the figure. As shown previously (Fig. 1), mutant TCO439 was highly attenuated for GI infection (A) (TCO439 compared to WT by both gavage and rectal inoculation, p < 0.0001).
Gastrointestinal infection (by gavage or intrarectal inoculation) with WT conferred striking protective immunity (decreased bacterial shedding and shorter duration of infection) to vaginal challenge, whereas neither rectal nor gavage inoculation of TC0439 protected against vaginal challenge \( p < 0.0005 \) (B). All mice receiving GI (rectal or gavage) WT infection were protected from the developed hydrosalpinx upon vaginal challenge.

**Figure 5.** Antibody response of TC0439 GI-infected mice increases following vaginal rechallenge. Sera were collected from the WT and TC0439 infected mice described in Figure 4 and analyzed by EB ELISA. Antibody responses are presented as mean log$_2$ titers + SD for each of the indicated Ig classes. Dashed line indicates cut-off values for positive serologic response. Anti-chlamydia antibody response of rectal (A) or gavage (C) challenged mice at 146 days following GI challenge. Sera were collected 21 days following vaginal rechallenge of rectal infected (B) or gavage infected (D) mice, and analyzed by EB ELISA. WT GI infection (both rectal and gavage infection) elicited robust anti-chlamydia antibody responses, and titers did not significantly change upon vaginal rechallenge \( p > 0.05 \). TC0439 elicited significantly lower anti-chlamydial antibody responses than WT \( p < 0.032 \) for IgG1, IgG2b, IgG2c, and IgA, but not for IgG3 following either primary rectal or gavage GI infection (panels A and C), and antibody responses following vaginal challenge rose to levels comparable to WT (rectal challenge panel B, \( p > 0.05 \) for all Ig class and subclass responses; gavage challenge panel D, \( p < 0.032 \) IgG2b, IgG2c and IgA, and \( p > 0.05 \) for IgG1 and IgG3).

**Figure 6.** ID$_{50}$ of TC0439 for GI infection is 1000-fold greater than WT. Mice were challenged intrarectally with the indicated doses of either WT or TC0439, and infection was followed by swabbing the rectum and enumerating IFUs on HeLa cell monolayers. Data are presented as geometric mean ± SD at day 21 post challenge. The calculated ID$_{50}$ for WT is $10^{2.97}$ (95% confidence interval 2.23 to 3.65) and for TC0439 is $10^{6.85}$ (95% confidence interval 6.18 to 7.59), \( p < 0.0003 \).
Figure 7. *C. muridarum* strains with nonsense mutations in *tc0600* are highly attenuated for GI infection. (A). Mice were pretreated with Depo-Provera and challenged with 5 X 10^4 IFUs of *C. muridarum* WT (n=5), GuaB (n=10) or TC0437/0439 (n=5). Vaginal vaults were swabbed at the indicated times and IFUs were enumerated on HeLa cell monolayers by immunofluorescence. Data are presented as geometric mean ± SD of IFUs recovered at the indicated days post-infection. Both mutants displayed vaginal infections similar to WT, although somewhat lower levels of chlamydial shedding of the mutants were observed compared to WT. The overall interaction effect between strains was not different (p > 0.22). However, tests comparing mutant GuaB and TC0437/0439 and WT main effects was statistically significant (p < 0.005). (B) Mice were inoculated intra-rectally with 10μl containing 1 X 10^5 IFUs of either WT (n=8), GuaB (n=10) or TC0437/0439 (n=8). Infection was assessed weekly by swabbing the rectum and counting IFUs on HeLa cell monolayers. Data are presented as geometric mean ± SD of IFUs at the indicated time points. The overall interaction effect between the strains was not statistically significant (p > 0.11), however tests comparing GuaB to WT, TC0437/0439 to WT, and GuaB to TC0437/0439 main effects were significant (p < 0.0001, p < 0.014, and p < 0.0006, respectively).

Figure 8. Venn diagram of the missense and nonsense mutations found in TC0439 and GuaB. *, depicts a stop codon.
**TABLE 1. Summary of SNPs in *C. muridarum* mutants**

| Strain     | Total number of mutations | Silent mutations | Missense mutations | Intergenic mutations | Nonsense mutations |
|------------|---------------------------|------------------|-------------------|----------------------|--------------------|
| TC0437     | 44                        | 13               | 29                | 0                    | tc0412 473585 C -> T, tc0437 506777 C -> T, Q -> STP |
| TC0438     | 35                        | 6                | 26                | 0                    | tc0412 473585 C -> T, tc0438 516403 C -> T, tc0450 549083 C -> T, Q -> STP |
| TC0439     | 28                        | 10               | 13                | 3                    | tc0439 527311 C -> T, tc0600 717761 G -> A, Q -> STP |
| TC0437/0439| 53                        | 15               | 35                | 0                    | tc0412 473585 C -> T, tc0437 506777 C -> T, tc0439 526979 G -> A, Q -> STP |
| GuaB       | 29                        | 8                | 17                | 2                    | tc0443 541698 G -> A, tc0600 717761 G -> A, Q -> STP |
Table 2. Mutations present in both TC0439 and GuaB

| Genomic Position | Gene ID  | Description                                      | Nucleotide change | Amino acid change |
|------------------|----------|--------------------------------------------------|-------------------|-------------------|
| 62823            | tc0054   | penicillin binding protein                       | G -> A            | Gly -> Glu        |
| 126188           | intergenic between tc0106 + tc0107 | intergenic        | C -> T            | Intergenic        |
| 224779           | tc0191   | hypothetical protein                             | G -> A            | Gly -> Gly        |
| 233168           | tc0197   | polymorphic membrane protein, pmpD               | G -> A            | Glu -> Lys        |
| 292616           | tc0250   | hypothetical protein                             | C -> T            | Val -> Ile        |
| 349120           | tc0290   | hypothetical protein                             | G -> A            | Ser -> Phe        |
| 369812           | tc0312   | glycosyl hydrolase, glgX                        | G -> A            | Ser -> Phe        |
| 573799           | tc0473   | peptide ABC transporter, permease protein        | C -> T            | Pro -> Leu        |
| 717761           | tc0600   | hypothetical protein                             | G -> A            | Gln -> STP        |
Titer Log$_{2}$

| Group        | IgG1 | IgG2b | IgG2c | IgG3 | IgA |
|--------------|------|-------|-------|------|-----|
| TC0439 (n=6) |      |       |       |      |     |
| WT (n=6)     |      |       |       |      |     |
| TC0437 (n=6) |      |       |       |      |     |
| TC0438 (n=6) |      |       |       |      |     |
A. Rectal

B. Gavage

Titer Log₂

TC0437 (n=4)
TC0438 (n=4)
TC0439 (n=5)
WT (n=4)
A. Primary GI Infection

B. Vaginal Rechallenge of GI Infected Mice

- TC0439 rectal (n=5)
- WT rectal (n=5)
- TC0439 gavage (n=5)
- WT gavage (n=4)
A. Vaginal

- GuaB (n=10)
- TC0437/439 (n=5)
- WT (n=5)

Day Post Infection vs Log10 IFU
