

RESEARCH ARTICLE

Plasmid deficiency in urogenital isolates of *Chlamydia trachomatis* reduces infectivity and virulence in a mouse model

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In this paper further evidence of the important role of the chlamydial plasmid in virulence is provided. In both the respiratory and urogenital tract sites, the plasmid-containing version of *Chlamydia trachomatis* was more virulent than the non-plasmid strain, when analysed in the mouse model. While this has previously been shown with the mouse strain of *Chlamydia*, this is the first report showing similar virulence characteristics of the human *C. trachomatis* strains.

Keywords

Chlamydia; plasmid; mouse; virulence; infection; genome.

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Introduction

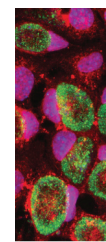
Chlamydial plasmids are all small [c. 7.5 kb, 8 coding sequences (CDSs)], highly conserved (< 1% variation within the species), nonconjugative, and nonintegrative, and their role in *C. trachomatis* pathobiology remains to be fully explored (Thomas *et al.*, 1997; Seth-Smith *et al.*, 2009). A

Abstract

We hypothesized that the plasmid of urogenital isolates of *Chlamydia trachomatis* would modulate infectivity and virulence in a mouse model. To test this hypothesis, we infected female mice in the respiratory or urogenital tract with graded doses of a human urogenital isolate of *C. trachomatis*, serovar F, possessing the cognate plasmid. For comparison, we inoculated mice with a plasmid-free serovar F isolate. Following urogenital inoculation, the plasmid-free isolate displayed significantly reduced infectivity compared with the wild-type strain with the latter yielding a 17-fold lower infectious dose to yield 50% infection. When inoculated via the respiratory tract, the plasmid-free isolate exhibited reduced infectivity and virulence (as measured by weight change) when compared to the wild-type isolate. Further, differences in infectivity, but not in virulence were observed in a *C. trachomatis*, serovar E isolate with a deletion within the plasmid coding sequence 1 when compared to a serovar E isolate with no mutations in the plasmid. We conclude that plasmid loss reduces virulence and infectivity in this mouse model. These findings further support a role for the chlamydial plasmid in infectivity and virulence *in vivo*.

long-standing observation has been that naturally occurring plasmid-deficient *C. trachomatis* are exceedingly rare, implying a significant, but noncritical role in fitness and transmission *in vivo*.

In vivo studies of the *C. trachomatis* plasmid have only recently begun and have thus far employed either the plasmid-free human *C. trachomatis* lymphogranuloma



venereum (LGV, serovar L2, strain 25667R) or a plasmid-free trachoma isolate (serovar A, strain A2497P-) where the plasmid has been cured by novobiocin treatment (Carlson *et al.*, 2008; Olivares-Zavaleta *et al.*, 2010; Kari *et al.*, 2011). In ocular infection of lower primates, plasmid-free *C. trachomatis* strain A2497P- was found to infect, but was significantly attenuated for shedding of viable chlamydiae and inducing inflammatory responses (Kari *et al.*, 2011). In mice inoculated in the lower urogenital tract with a naturally occurring plasmid-free isolate of *C. trachomatis* LGV, serovar L2, reduced infectivity was seen (Carlson *et al.*, 2008; Olivares-Zavaleta *et al.*, 2010). It is important to note that in these studies, the plasmid-free *C. trachomatis* strains, while attenuated for infectivity in the mouse urogenital tract (L2, 25667R) or in primate eyes (A, A2497P-), nonetheless were able to elicit immune responses that protected against virulent and fully infectious matched plasmid-containing wild-type strains. This has led some to assert that plasmid-free strains may be effective attenuated vaccines (Olivares-Zavaleta *et al.*, 2010; Kari *et al.*, 2011). Similar results have been reported in the mouse model of urogenital infection with the mouse pathogen, *C. muridarum* (O'Connell *et al.*, 2007). It is also important to note that none of the aforementioned studies have utilized urogenital isolates of *C. trachomatis*, and thus, it could be hypothesized that the plasmid of urogenital isolates possesses different qualities and would behave differently in an animal model when compared to the others thus far examined.

In the present study, we have assessed for the first time the infectivity and virulence of naturally occurring plasmid-free and a cocirculating plasmid-containing (hereafter, wild-type) urogenital isolates of *C. trachomatis*, serovar F, as well as the Swedish new variant *C. trachomatis* (nvCT; serovar E, with a mutation in CDS1) in a mouse model of urogenital or respiratory infection.

Materials and methods

Chlamydia trachomatis strains used in this study

Four urogenital isolates of *C. trachomatis* were used in this study; *C. trachomatis* Sweden 3 and 4 (hereafter, E/SW3 and F/SW4, respectively) were isolated in Malmö, Southern Sweden, in 2002 and are serovar E and F, respectively, representing plasmid-containing wild-type cervical isolates (Seth-Smith *et al.*, 2009). *Chlamydia trachomatis* Sweden 2 (E/SW2), representing the Swedish nvCT (plasmid-containing serovar E, with a 377-base pair deletion in CDS1), was isolated from a male urethra in Malmö in 2006 (Seth-Smith *et al.*, 2009; Unemo *et al.*, 2010). *Chlamydia trachomatis* Sweden, serovar F, plasmid-free (F/SWFP-) is a cervical isolate from Malmö, Sweden, in 1995 (Persson *et al.*, 1996; Wang *et al.*, 2012). All isolates were cultured only in McCoy cells with minimal passage number.

Genome sequencing

Extraction of DNA was performed on strain F/SWFP- as previously described (Seth-Smith *et al.*, 2009). DNA was

sequenced on the Illumina GAI platform using 54-bp paired-end reads, resulting in a mean genome coverage of $1527 \times$. A subset of Illumina reads was assembled using Velvet, version 1.0.12 (Zerbino & Birney, 2008), producing contiguous nucleotide sequences (contigs), which were scaffolded against the genome of strain F/SW4 (EMBL accession no. HE601804) (Harris *et al.*, 2012), using ABACAS (Assefa *et al.*, 2009). The two rRNA operons assembled into a single contigs, which were manually corrected, as were contig boundaries where possible. Manual insertion of capillary-sequenced *hctB* and *tarp* gene sequences was followed by self-mapping with all the sequence reads to confirm the assembled sequence and resulted in an improved high-quality draft sequence (Chain *et al.*, 2009). After this process, the assembly of strain F/SWFP- comprised three contigs. Annotated features were transferred from the closely related strain F/SW4 using annotations_update.pl (https://github.com/sanger-pathogens/annotations_update) on the basis of BLASTN similarity using the default cut-off of 90% and manually curated using Artemis (Rutherford *et al.*, 2000). Mapping of sequence data to reference genomes was carried out using SMALT (<http://www.sanger.ac.uk/resources/software/smalt/>). Genome comparisons were visualized using the Artemis Comparison Tool (ACT) (Carver *et al.*, 2005). The genome sequence and annotation have been deposited in the EMBL database under the accession number HE605380, and the read data are in SRA under the accession number ERS001400.

Mice

We selected an outbred strain of mice (Swiss Webster, SW) for these experiments. Five- to six-week-old female SW mice were obtained from Harlan Sprague Dawley (Indianapolis, IN) and housed with commercial rodent chow and water *ad libitum* with a 12 : 12 light/dark cycle. All mice were subject to a 7–10-day facility acclimation period prior to inclusion in the study. All studies were approved by the Midwestern University Institute Animal Care and Use Committee (MWU File # 1726).

Infection of mice and assessment of infection by culture

For urogenital infection, mice were pretreated with 2.5 mg of medroxyprogesterone acetate (DepoProvera, P4; Greenstone LLC, Peapack, New Jersey) and 7 days later were inoculated intravaginally (i.vag.) with \log_{10} -graded doses of McCoy cell-grown *C. trachomatis* as described elsewhere (Ramsey *et al.*, 1999). To verify and track urogenital infection, cervical–vaginal swabs were collected in all mice at day 4, 7, 10, and 14 postinfection in each experiment. *Chlamydia trachomatis* was isolated inclusions visualized by indirect immunofluorescence and quantified as inclusion-forming units (IFU) in HeLa 229 cultures as previously described (Ramsey *et al.*, 1999).

For respiratory infection, mice were anesthetized via inhalation of 4–5% isoflurane and placed in dorsal recumbency, and \log_{10} -graded doses of the strains were inoculated

dropwise into the nasal nares exactly as described elsewhere (Ramsey *et al.*, 2009). Mice were weighed daily, and weight gain or loss from baseline weight was recorded as a measure of morbidity. It should be noted that none of the inoculated mice exhibited any overt morbidity from the infection, and there were no mortalities following infection. Hence, lethal dose that induces mortality in 50% of the inoculated mice (LD₅₀) was not determined.

Antibody responses

For both urogenital and respiratory inoculations, we have found it useful to assess seroconversion postinfection as a means to assess infection at levels below the level of detection of our culture methods (e.g. at low dose infection). For this purpose, blood was collected at day 0, just prior to infection, and 35 or more days postinoculation, and total plasma anti- *C. trachomatis* immunoglobulin G (IgG) levels were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (Ramsey *et al.*, 1989, 2009). An antibody titer ≥ 40 was considered positive for the determination of seroconversion. Immunoglobulin G antibody responses were also compared as a measure of elicitation of adaptive immunity and as an indirect measure of the degree of infection (greater infectious burden correlating to a greater antibody response). For quantitative comparison of antibody responses, titers were converted to log₁₀, and the arithmetic mean of all seropositive animals was determined as described elsewhere (Ramsey *et al.*, 1988).

Statistics

Infection course data, as assessed by shedding of viable organisms (lower genital tract) and weight change over time

(respiratory tract), were compared by analysis of the variance (ANOVA, treatment group, days) with repeated measures. Results for ELISA and IFU counts at individual time points were compared using a two-tailed unpaired *t*-test. The incidence of viable chlamydial shedding and seroconversion was assessed using Fisher's exact test. Infectious dose that yielded a 50% of mice infected (ID₅₀) was estimated by the method of Reed and Muench (Reed & Muench, 1938).

Results

Plasmid deficiency in a *C. trachomatis* urogenital isolate correlates with reduced infectivity in the mouse urogenital tract

In our first experiment, we intravaginally inoculated groups of 10 mice with log₁₀-graded doses of either F/SW4 or F/SWFP– *C. trachomatis* and monitored outcomes of infection. Table 1 shows the results of the study. In both wild-type and plasmid-free inoculated groups, low numbers of viable organisms were shed intermittently through day 10 postinfection, but no animals in either group remained culture positive by day 14. The wild-type F/SW4 isolate had an estimated ID₅₀ of 3.2×10^5 IFU. We were unable to infect 50% of the mice with the plasmid-free isolate using our highest dose of 10⁶ IFU – despite a second experimental attempt using 7 additional mice (data compiled in Table 1). Thus, we assert only that the ID₅₀ of the F/SWFP– isolate (as determined by culture) to be in excess of 10⁶ IFU. In addition, at the 10⁶ IFU dose, the day 4 and day 7, IFU isolation attempts indicated a greater infectious burden in animals inoculated with the F/SW4 isolate ($P = 0.01$ and $P = 0.03$, respectively, by two-tailed unpaired *t*-test). As we

Table 1 ID₅₀ determination with Serovar F plasmid variant strains in urogenital tract infection

Dose	F/SW4 (plasmid +)		F/SWFP (plasmid –)		<i>P</i> -value*
	Culture + [†]	Seroconversion [‡] (mean Ab titer ± SD)	Culture + [†]	Seroconversion [‡] (mean Ab titer ± SD)	
10 ³	0/10	5/10 (1.08 ± 1.0)	0/10	2/10 (0.76 ± 1.1)	0.35
10 ⁴	0/10	6/10 (1.4 ± 1.4)	0/10	5/10 (0.8 ± 0.87)	1.0
10 ⁵	3/10 (40–484)	9/10 (2.3 ± 0.8)	0/9	5/9 (1.93 ± 0.64)	0.22/0.15
10 ⁶	7/10 (16–2705)	10/10 (2.51 ± 0.8)	8/17 (4–192)	12/17 (1.8 ± 0.48)	0.42/0.12
ID ₅₀ [§]	316 277	2956	> 10 ⁶	50 133	

**P* values were determined by comparing strains at each dose for either the ratio of culture positive to culture negative or the ratio of seroconverters by a two-tailed Fisher's exact test. Geometric mean antibody titers were compared by a two-tailed Student's *t*-test.

[†]Number of mice culture positive of the total mice inoculated at the given dose with either F/SW4 or F/SWFP– strains. Culture detection of infection was attempted on swabs collected at 4, 7, and 10 days postinfection. Range of IFU counts (considering all mice and time points postinfection) is shown in parentheses.

[‡]Number of mice of the total tested displaying seroconversion for plasma IgG antibody (day 35 postinfection) against serovar F antigen. The mean geometric antibody titer ± the standard deviation of mice that seroconverted following inoculation is provided in parentheses (titers ranged from 40 to 5120).

[§]ID₅₀ is the calculated dose that will achieve 50% infection rate. Data are given in IFUs as calculated by the method of Reed and Muench (Reed & Muench, 1938).

have previously observed, seroconversion provides a more sensitive method of determining infection below the level of detection of culture (Ramsey *et al.*, 2009). Thus, we were able to use seroconversion to achieve an ID₅₀ for both groups with the plasmid-free isolate also displaying reduced infectivity (ID₅₀ ~ 17-fold higher) by this measure. Antibody titers were not statistically different between the two groups. Cumulatively, these data show that while the mouse urogenital tract is not a highly permissive environment for urogenital isolates of *C. trachomatis*, plasmid deficiency renders the pathogen even less fit when compared to a wild-type serovar-matched isolate in this model.

Plasmid deficiency in a *C. trachomatis* urogenital isolate correlates with reduced infectivity and morbidity in the mouse respiratory tract

Unlike *C. muridarum*, which naturally ascends into the upper urogenital tract and induces pathogenic host responses, *C. trachomatis* isolates are less likely to ascend and are less virulent for the urogenital tract in the mouse model. Induction of chronic oviduct damage by *C. trachomatis* in the mouse requires direct inoculation into the upper genital tract of mice via surgical exposure of the ovarian bursa or oviducts (Tuffrey *et al.*, 1986a, b). However, weight change following respiratory infection is a common means to generally assess human pathogen virulence in rodent models (Fox, *et al.* Ed., 2007) and has been used to assess chlamydial virulence in the mouse (Ramsey *et al.*, 2009; He *et al.*, 2011). For this reason, we sought to assess the *in vivo* infectivity and morbidity of F/SWFP– and wild-type F/SW4 *C. trachomatis* via intranasal inoculation of mice.

Table 2 shows that statistically significant differences in infectivity were observed between the groups at the lowest dose where 4 of 10 animals seroconverted when inoculated

with 10³ IFU of the F/SWFP– (estimated ID₅₀ = 1292 IFU), but all 10 animals seroconverted when inoculated with plasmid-carrying F/SW4 (ID₅₀ < 1000) at the same dose. Statistical significance was also achieved when assessing differences in antibody levels in the groups inoculated with 10⁶ IFU of the wild-type strain evoking higher antibody levels in those that seroconverted. While not consistent across all doses, these data indicate enhanced infectivity in the presence of the plasmid.

Figure 1 shows that following an initial loss in weight in all groups (likely due to the stress of anesthesia and dosing procedures), all groups gradually recovered initial body weight and began to gain weight above baseline day 0 weights. Overall, a distinct trend was observed in the 10³, 10⁴, and 10⁵ groups where mice inoculated with the wild-type F/SW4 strain gained less weight over time than those inoculated with identical doses of the F/SWFP– isolate. While this did not achieve statistical significance at the 10³ dose – likely due to low number of the F/SWFP– isolate-infected mice with only 4 mice in this group seroconverting – the difference in weight gain in the 10⁴ and 10⁵ groups was highly significant. This indicates that the F/SW4 isolate caused greater morbidity than did the plasmid-deficient F/SWFP– isolate. Interestingly, at the highest intranasal dose (10⁶), no differences in weight gain over time between the wild-type and plasmid-free infected groups were observed, although both groups gained less weight than identical groups at lower doses. This suggests that higher doses compensate for the loss of the plasmid when comparing weight changes observed in the lower dose groups. Lastly, it is notable that neither the plasmid-free *C. trachomatis* F/SWFP– nor the F/SW4 isolate induced any mortalities over the 21-day monitoring period, indicating low virulence of human urogenital *C. trachomatis* isolates in this nonhuman host.

Table 2 ID₅₀ determination with Serovar F plasmid variants in respiratory tract infection

Dose	F/SW4 (plasmid +)		F/SWFP (plasmid –)		P-value*
	Mortality [†]	Seroconversion [‡] (mean Ab titer ± SD)	Mortality	Seroconversion (mean Ab titer ± SD)	
10 ³	0/10	10/10 (1.99 ± 0.57)	0/10	4/10 (1.96 ± 0.7)	0.03
10 ⁴	0/10	10/10 (2.77 ± 0.58)	0/10	10/10 (2.75 ± 0.71)	1.0
10 ⁵	0/10	10/10 (3.51 ± 0.54)	0/10	10/10 (3.2 ± 0.38)	1.0
10 ⁶	0/10	10/10 (3.2 ± 0.35)	0/10	10/10 (3.62 ± 0.45)	1.0
ID ₅₀ [§]	ND	< 1000	ND	1292	0.03

*P values were determined by comparing strains at each dose for either the ratio of seroconverters by a two-tailed Fisher's exact test or geometric mean antibody titers by a two-tailed Student's *t*-test.

[†]Number of moribund mice of the total inoculated at the given dose with either F/SW4 strain or F/SWFP– strain through a *c.* 20-day monitoring period.

[‡]Number of mice of the total tested displaying seroconversion for plasma IgG antibody (day 35 postinfection) against serovar F (SW4) antigen. The mean geometric antibody titer ± the standard deviation of mice that seroconverted following inoculation is provided in parentheses (titer range = 20–10 240).

[§]Calculated infectious dose that will achieve 50% infection rate (seroconversion) as calculated by the method of Reed and Muench (Reed & Muench, 1938).

A naturally occurring plasmid mutation in CDS1 results in reduced infectivity without affecting virulence

In 2006, the Swedish nvCT that contained a 377-bp deletion mutation in CDS1 was isolated in Sweden (Ripa & Nilsson, 2006). One serovar E isolate from this outbreak, Sweden 2 (E/SW2), like all nvCT strains, was undetectable by two of the major nucleic acid-based diagnostic test targeting CDS1. Because this mutant pathogen amplified rapidly in the Swedish population, it was hypothesized that evolutionary pressures exerted by screen-and-treat public health programs using the diagnostic test had selected the mutant. However, it could not be ruled out that the degree of spread could also have been attributed to selective biological fitness advantages incurred as a result of plasmid deficiency. This is why, we sought to test the hypothesis that mutation in the plasmid CDS1 observed in E/SW2 enhanced *in vivo* fitness when compared to a closely matched and cocirculating

serovar E (E/SW3) isolate that carried a wild-type intact plasmid.

Table 3 shows the results of comparing infectivity of these two isolates in the urogenital tract following intravaginal inoculation. When assessed by culture, neither the wild-type nor plasmid mutant showed a difference in ID₅₀ or chlamydial shedding. However, there were slight differences in the rate of seroconversion with *c.* twofold higher ID₅₀ in the nvCT isolate E/SW2 and significantly increased antibody responses in the E/SW3 isolate when compared to the mutant at the 10⁵ and 10⁶ doses. Table 4 shows the results of comparing infectivity of the E/SW2 and E/SW3 isolates in the respiratory tract following intranasal inoculation. As seen with urogenital infection, the ID₅₀ was increased in the plasmid mutants over the wild-type isolate. However, an accurate ID₅₀ for the E/SW3 infected group could not be obtained because, even at the lowest dose of 10³ IFU, all mice seroconverted, whereas at the identical dose of the

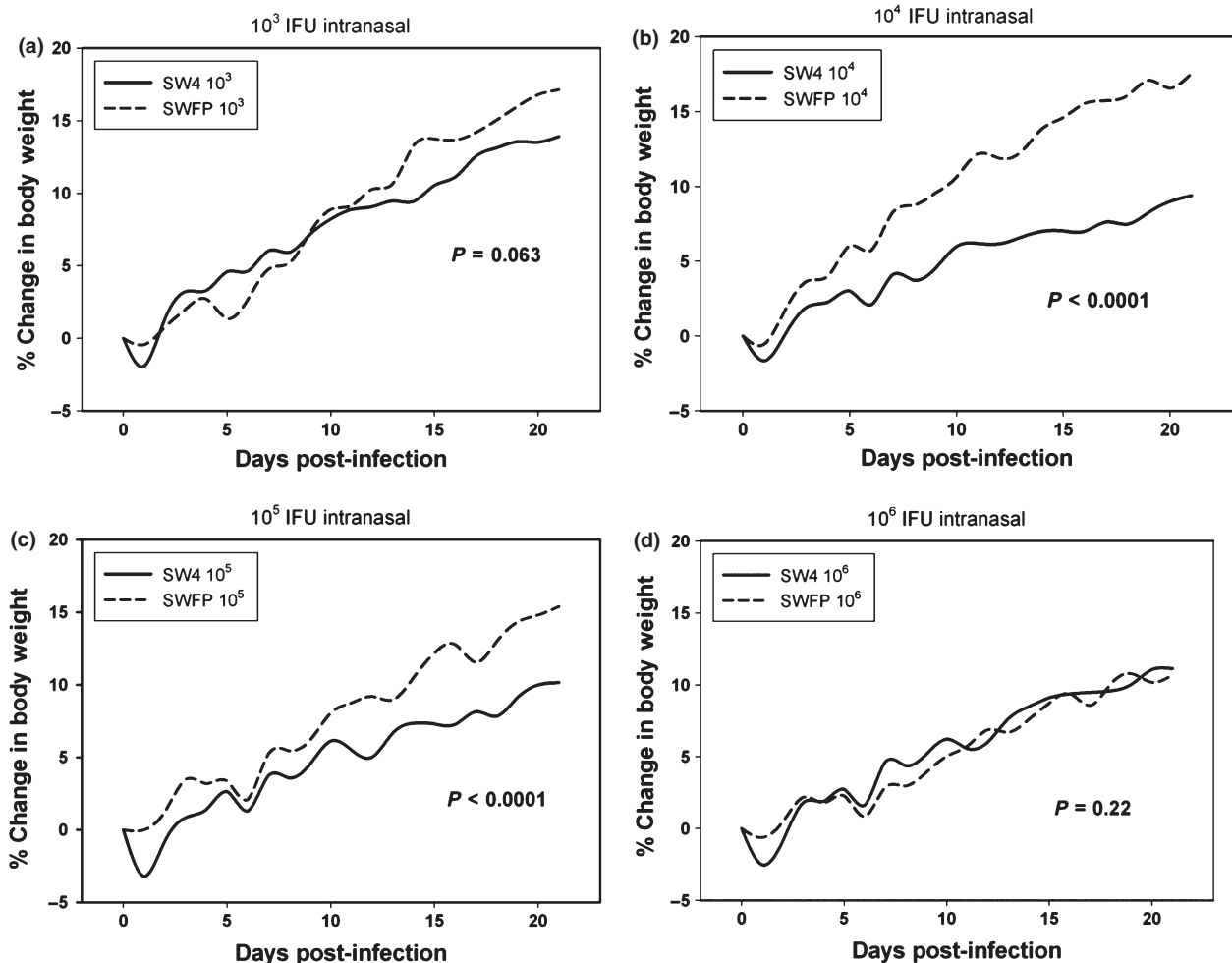


Fig. 1 Weight change following intranasal inoculation of mice with plasmid-deficient or plasmid-sufficient isolates of *Chlamydia trachomatis*, serovar F. Mice were administered graded doses of *C. trachomatis* serovar F urogenital isolates either F/SWFP-(plasmid-deficient) or F/SW4 (plasmid-sufficient). (a) 10³ IFU; (b) 10⁴ IFU; (c) 10⁵ IFU; and (d) 10⁶ IFU. *P* values shown are result of two-way, repeated-measures ANOVA. Each data point is the mean for all infected animals in that group (as indicated by seroconversion). For all groups, *N* = 10 with the exception of the group administered 10³ IFU of SWFP. In this case, only the results of the 4 mice that seroconverted following inoculation are shown.

Table 3 ID₅₀ determination with Serovar E plasmid variant strains in urogenital tract infection

Dose	E/SW3 (plasmid +)		E/SW2 (plasmid mutant)		P-value*
	Culture [†] (range of IFU)	Seroconversion [‡] (mean Ab titer ± SD)	Culture [†] (range of IFU)	Seroconversion [‡] (mean Ab titer ± SD)	
10 ³	0/10	7/10 (2.2 ± 0.65)	0/10	6/10 (2.2 ± 0.6)	1.0
10 ⁴	0/10	8/10 (1.7 ± 1.0)	0/10	7/10 (2.2 ± 0.3)	1.0
10 ⁵	5/10 (30–520)	10/10 (2.32 ± 0.6)	5/10 (30–160)	9/10 (1.7 ± 0.66)	1.0
10 ⁶	10/10 (200–5260)	10/10 (2.96 ± 0.61)	10/10 (30–4070)	10/10 (2.32 ± 0.67)	1.0
ID ₅₀ [§]	100 000	718	100 000	1631	0.04

*P values were determined by comparing strains at each dose for either the ratio of culture positive to culture negative or the ratio of seroconverters by a two-tailed Fisher's exact test. Geometric mean antibody titers were compared by a two-sided Student's *t*-test.

†Number of mice culture positive of the total mice inoculated at the given dose with either E/SW3 or E/SW2 strain. Culture detection of infection was attempted on swabs collected at 4, 7, and 10 days postinfection. Range of IFU counts (considering all mice and time points postinfection) are shown in parentheses.

‡Number of mice of the total tested displaying seroconversion for plasma IgG antibody (day 35 postinfection) against serovar E antigen. The mean geometric antibody titer ± the standard deviation of mice that seroconverted following inoculation is provided in parentheses (titers ranged from 40 to 5120).

§ID₅₀ is the calculated dose that will achieve 50% infection rate. Data are given in IFUs as calculated by the method of Reed and Muench (Reed & Muench, 1938).

Table 4 ID₅₀ determination with Serovar E plasmid variant strains in respiratory tract infection

Dose	E/SW3 (plasmid +)		E/SW2 (plasmid mutant)		P-value*
	Mortality [†]	Seroconversion [‡] (mean Ab titer ± SD)	Mortality [†]	Seroconversion [‡] (mean Ab titer ± SD)	
10 ³	0/10	10/10 (2.2 ± 0.48)	0/10	4/10 (2.8 ± 1.0)	0.02
10 ⁴	0/10	10/10 (3.3 ± 0.48)	0/10	9/10 (2.2 ± 0.58)	0.85
10 ⁵	0/10	10/10 (3.65 ± 0.28)	0/10	10/10 (3.38 ± 0.39)	0.0008
10 ⁶	0/10	10/10 (3.77 ± 0.31)	0/10	10/10 (3.5 ± 0.25)	1.0
ID ₅₀ [§]		< 1000		1743	0.046

*P values were determined by comparing strains at each dose for either the ratio of seroconverters by a two-tailed Fisher's exact test or geometric mean antibody titers by a two-tailed Student's *t*-test.

†Number of moribund mice of the total inoculated at the given dose with either E/SW3 (plasmid-intact parental) strain or E/SW2 (plasmid mutant) strain through a c. 20-day monitoring period.

‡Number of mice of the total tested displaying seroconversion for plasma IgG antibody (day 35 postinfection) against serovar E antigen. The mean geometric antibody titer ± the standard deviation of mice that seroconverted following inoculation is provided in parentheses (titer range = 40–10 240).

§Calculated infectious dose that will achieve 50% infection rate (seroconversion) as calculated by the method of Reed and Muench (Reed & Muench, 1938).

plasmid mutant E/SW2, only 4 of 10 seroconverted. Additionally, as seen with urogenital infection, intranasal infection with the wild-type isolate stimulated higher antibody responses at the 10⁴ and 10⁶ doses, while the difference noted at the 10⁵ dose did not quite achieve statistical significance. Weight change post-intra-nasal infection was also monitored in this group, but there were no remarkable differences in weight loss or reduction in weight gain over time as was seen following intranasal infection with the mutant-isolate in the previous experiments (data not

shown). From these data, we conclude the mutation that occurred in the E/SW2 isolate had a modest effect on *in vivo* fitness of the isolate in the mouse host, and this effect was to reduce fitness, not to enhance it.

Genome comparisons

The genomes of nvCT serovar E strains E/SW2 and contemporaneous E/SW3 and E/SW4 have been described and published elsewhere (Harris *et al.*, 2012). When

comparing nvCT strain E/SW2 and E/SW3, we previously showed that there were a total of 229 SNPs separating these two strains, 158 of which are found in a 30-kb region spanning genes SW2 1391 (CTL-393) to SW2 1601 (CTL-417) (Harris *et al.*, 2012). This SNP-dense region was shown to be a region of recombination and encompasses genes located alongside the plasticity zone or PZ. The PZ is a region of high variation both in terms of recombination and functional gene loss.

As a comparator to F/SW4 for our *in vivo* studies, the genome of strain F/SWFP– was sequenced and assembled. The F/SWFP– genome was 1 042 732 bp in length with no evidence of plasmid, either as an extrachromosomal element or integrated into the chromosome. There were no wholesale gene gains or losses. In addition to the lack of plasmid, the genome of F/SWFP– also differed from that of strain F/SW4 by 178 SNPs listed in Supporting Information, Table S2. The majority of these were found clustered in two regions likely to be explained by recombination. 31 SNPs between bases 167 543 and 171 733 of the F/SW4 genome, 26 being located within gene F/SW-41481 predicted to encode an integral membrane protein and a single SNP in F/SW-41491 – a FAD-dependent monooxygenase. Notably, this region overlaps with the putative recombinant regions in nvCT serovar E strain E/SW2 (Harris *et al.*, 2012). The second region of high SNP density contained 92 SNPs found between bases 710 211 and 725 729, affecting a 15-kb region encompassing 14 genes, F/SW4 6341–6471. The affected genes fall into several functional classes including core functions as well as more those orthologous to putative virulence genes. One example is the *mviN* gene, which has been shown to play a role in virulence in *Francisella tularensis* infections in mice (Ulland *et al.*, 2010). Analysis of the pseudogenes within F/SWFP– did not indicate any unique pseudogenes compared with other sequenced strains of serotype E and F (F/SW4, E/SW2, E/SW3; Table S3).

Discussion

For many years, the chlamydial plasmid was referred to as the 'cryptic plasmid of *Chlamydia*'. This descriptor referenced the unknown function of the plasmid that is carried by nearly all *C. trachomatis* ocular and urogenital isolates. However, it is arguably no longer accurate to refer it as 'cryptic'. For example, we now know the criticality of the plasmid in eliciting pathogenic immune responses in several model systems as well as in conveying certain key *in vitro* phenotypes (e.g. glycogen accumulation) and in controlling the expression of certain genes on the *Chlamydia* chromosome. Overall, studies of infection with both wild-type and plasmid-free *C. trachomatis* and *C. muridarum* strains in mouse urogenital infection indicate that the plasmid modulates infectivity and may be essential for pathogenic immune responses (O'Connell *et al.*, 2007; Carlson *et al.*, 2008; Olivares-Zavaleta *et al.*, 2010; Russell *et al.*, 2011). It is all but certain that either *C. muridarum* plasmid gene products or products of plasmid responsive loci on the bacterial chromosome engage Toll-like receptor-2 (TLR-2) causing

deleterious responses in mouse urogenital infections with *C. muridarum* (O'Connell *et al.*, 2011). Similarly, in ocular infection of lower primates, plasmid-free *C. trachomatis* was found to infect, but was significantly attenuated for inducing inflammatory responses (Kari *et al.*, 2011). Although the plasmid seems essential for certain measures of pathogenicity in mouse urogenital tract infection with *C. muridarum*, in the mouse respiratory tract, deficiency of the plasmid in *C. muridarum* seems to have opposite effects, with increased pathological responses when the plasmid is not present (He *et al.*, 2011). Likewise, it is important to note that plasmid-free *C. psittaci* intraperitoneal (i.p.) infections of mice or plasmid-free *C. caviae* urogenital infection in guinea pigs results in different outcomes (Miyairi *et al.*, 2011; Frazer *et al.*, 2012). In both models, the plasmid was dispensable regarding attenuation of virulence, and in the *C. psittaci* i.p. mouse model, a handful of chromosomal single nucleotide polymorphisms was left to account for virulence differences between wild-type and plasmid-free strains (Miyairi *et al.*, 2011). Interestingly, the important human and zoonotic pathogen, *C. pneumoniae*, does not normally carry a plasmid nor does its chromosome contain any genes related to chlamydial plasmids (Thomas *et al.*, 1997). It would be convenient if all observations of chlamydial strains and infections in various models were consistent, but one must remember that these differences likely reflect different chlamydial species and/or infection at a different anatomical site and thus adaption of the pathogen and its plasmid to each host and anatomical niche. Based on these observations, one could readily assert that each *Chlamydia*–host interaction is unique, and the role of the plasmid in these interactions is concomitantly variable.

In the present study, we have specifically assessed for the first time, the infectivity and virulence of naturally occurring wild-type and plasmid-free urogenital isolates of *C. trachomatis*, serovar F. Mice inoculated with a plasmid-free urogenital isolate of *C. trachomatis* displayed higher ID₅₀ by both urogenital and respiratory routes of inoculation and reduced weight gain over time. There were no wholesale gene deletions or substitutions in these isolates although several SNPs were observed – especially in selected regions of the chromosome. While we cannot rule out a contributory role of genes affected by SNPs in these isolates, our data are consistent overall with studies in other model systems that support a critical role for the plasmid in both infectivity and virulence in this model.

With regard to nvCT E/SW2, it was originally speculated that the mutation of the plasmid in nvCT (E/SW2) was responsible for amplification in the Swedish regional population due to either a diagnostic or biological selective advantage (Ripa & Nilsson, 2006; Unemo & Clarke, 2011). Further, according to one large clinical study, women infected with nvCT were less likely to exhibit symptomatic urethral infection and lower abdominal pain (Bjartling *et al.*, 2009). Transmission was rare among those with more international sexual networks such as heterosexuals in higher ages and men who have sex with men (Unemo & Clarke, 2011). Subsequently, no outstanding *in vitro* differences in nvCT were discerned by microscopy, growth

kinetics, cell tropism, antimicrobial susceptibility, or inclusion glycogen inclusion when compared to similar wild-type isolates including, a serovar E *C. trachomatis* predominating in Sweden prior to the isolation of the mutant (Unemo *et al.*, 2010; Unemo & Clarke, 2011). Likewise, in these studies, the genomic signature compared with other isolates was unremarkable with no wholesale gene deletions or substitutions. These results indicated that preponderance of this isolate in many regions of Sweden was likely due to a diagnostic selective advantage. Yet, fitness had heretofore not been directly tested *in vivo*.

In the present study, mice infected with nvCT E/SW2 displayed some moderately reduced parameters of infectivity, but measures of virulence in the respiratory tract were more equivocal. Overall, *in vivo* fitness appears reduced, and thus these data do not support the mutant's expansion as a result of *in vivo* fitness and thus would more likely support its expansion as a result of positive selective pressure from diagnostic test avoidance.

Some caution should be exercised in interpreting our present data. First of all, we carefully selected serovar-matched isolates that were predominating in the human population at or before the time the F/SWFP— or plasmid mutant E/SW2 was initially isolated (Unemo & Clarke, 2011). Genome sequencing has indicated that while the chromosomes of the comparator strains differ on the account of putative recombinations and SNPs, there were no other whole gene differences. However, this was not unexpected (Tables S1 and S2 (Unemo *et al.*, 2010). This analysis also showed how apparently closely matched, cocirculating strains carry a number of SNPs, some in genes putatively associated with virulence. It is not possible to predict whether any of these SNPs contributed directly to the differences we have observed in the mouse model. However, our observations are quite consistent with the aforementioned *in vivo* observations of plasmid-free strains in human disease (Bjartling *et al.*, 2009; Harris *et al.*, 2012) and in other animal models (O'Connell *et al.*, 2007; Carlson *et al.*, 2008; Olivares-Zavaleta *et al.*, 2010; Russell *et al.*, 2011). Thus, we feel the most logical inference is to attribute the differences seen to presence and function of the plasmid.

Secondly, *C. trachomatis*, unlike *C. muridarum*, is not a natural pathogen of the mouse. Consequently, *C. trachomatis* infection induces neither the degree of infection burden nor the pathology that is observed following *C. muridarum* urogenital or respiratory infections (Rank, 2006). Thus, one should be mindful of these host and pathogen differences when interpreting our data. Nonetheless, it is pleasing that our findings largely support and agree with those of others investigating the chlamydial plasmid in mouse urogenital tract infections with LGV strains either *C. muridarum* or *C. trachomatis* (O'Connell *et al.*, 2007; Carlson *et al.*, 2008; Olivares-Zavaleta *et al.*, 2010; Russell *et al.*, 2011). In the context of what has already been reported by others regarding the chlamydial plasmid and *in vivo* fitness, our results add to the growing body of evidence that in many hosts and at specific anatomical sites, the plasmid plays a role in modulating infectivity and

virulence, but this role is not universal. Now that a means to genetically manipulate chlamydiae and selectively mutate the plasmid has been elaborated and the function of the plasmid can be dissected and studied in greater detail (Wang *et al.*, 2011; Song *et al.*, 2013).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Single nucleotide polymorphisms (SNPs) in E/SW2 compared to E/SW3.

Table S2. Single nucleotide polymorphisms (SNPs) in F/SWFP compared to F/SW4.

Table S3. Pseudogenes in the genomes of the strains studied.