Floxed-Cassette Allelic Exchange Mutagenesis Enables Markerless Gene Deletion in *Chlamydia trachomatis* and Can Reverse Cassette-Induced Polar Effects

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**ABSTRACT** As obligate intracellular bacteria, *Chlamydia* spp. have evolved numerous, likely intricate, mechanisms to create and maintain a privileged intracellular niche. Recent progress in elucidating and characterizing these processes has been bolstered by the development of techniques enabling basic genetic tractability. Florescence-reported allelic exchange mutagenesis (FRAEM) couples chromosomal gene deletion with the insertion of a selection cassette encoding antibiotic resistance and green fluorescent protein (GFP). Similar to other bacteria, many chlamydial genes exist within polycistronic operons, raising the possibility of polar effects mediated by insertion cassettes. Indeed, FRAEM-mediated deletion of *Chlamydia trachomatis* tmeA negatively impacts the expression of tmeB. We have adapted FRAEM technology by employing a *gfp-bla* cassette flanked by *loxP* sites. Conditional expression of Cre recombinase in *Chlamydia tmeA* containing a floxed cassette resulted in deletion of the marker and restoration of tmeB expression.

**IMPORTANCE** *C. trachomatis* infections represent a significant burden to human health. The ability to genetically manipulate *Chlamydia* spp. is overcoming historic confounding barriers that have impeded rapid progress in understanding overall chlamydial pathogenesis. The current state of genetic manipulation in *Chlamydia* spp. requires further development, including mechanisms to generate markerless gene disruption. We leveraged a stepwise Cre-lox approach to excise selection marker genes from a deleted gene locus. We found this process to be efficient, and the removal of extraneous elements resulted in the reversal of a negative polar effect on a downstream gene. This technique facilitates a more direct assessment of gene function and adds to the *Chlamydia* molecular toolbox by facilitating the deletion of genes within operons.

**KEYWORDS** TmeA, type III secretion, FRAEM, mutagenesis, *Chlamydia*

The obligate intracellular bacterium *Chlamydia trachomatis* represents a significant burden to human health worldwide. Ocular infections with serovars A to C can lead to blinding trachoma, whereas genital infections mediated by serovars D to K or LGV1 to LGV3 result in typical sexually transmitted disease or lymphogranuloma venereum, respectively (1). *Chlamydia* spp. manifest a biphasic developmental cycle where infectious elementary bodies (EBs) and vegetative reticulate bodies (RBs) represent distinct developmental forms (2). Development occurs entirely within a parasitophorous vesicle termed an inclusion. Beginning as early as attachment and invasion, chlamydial orchestrate changes in host cell biology to create and maintain a productive growth environment. Major affected pathways include the host cytoskeleton, membrane and vesicle trafficking, host cell survival, and immune signaling (3).

Like other Gram-negative pathogens (4), *Chlamydia* spp. express a type III secretion system (T3SS) that secretes and translocates effector proteins (T3SE) whose antihost
activities culminate to create and maintain a hospitable intracellular niche (3). T3S activity occurs as early as invasion (5, 6) and likely continues until RBs differentiate back into EBs. Effectors that intercalate into the inclusion membrane (Incs) were first identified bioinformatically by the presence of a ca. 60-residue predicted hydrophobic domain (7) and represent the most thoroughly characterized group of chlamydial T3SEs. Surrogate T3SSs and protein localization studies have previously been leveraged to identify an additional pool of chlamydial effectors that are translocated beyond the inclusion membrane and target host proteins within the cytoplasm or organelles (3). The total number of T3S substrates likely exceeds 60 effectors (8), yet the functional role for the majority of these proteins remains to be elucidated.

Due to genetic intractability, it was historically difficult to provide definitive evidence regarding how chlamydial gene products contribute to development and pathogenesis; however, the acquisition of tools to genetically manipulate chlamydiae now provides opportunities to more efficiently reveal aspects of infection biology (reviewed in references 9 to 11). Early advances relied on elegant chemical mutagenesis strategies applied in forward and reverse genetic approaches to associate genes with particular phenotypes. The ability to coinfect chlamydial strains to exchange DNA via lateral gene transfer (LGT) was exploited for gene association studies in heavily mutagenized genomes (12). CaCl₂ chemical transformation of *C. trachomatis* (13) with exogenous plasmid DNA has led to the development of more targeted strategies, including use of the TargeTron system (14) and fluorescence-reported allelic exchange mutagenesis (FRAEM) (15). The TargeTron system enables site-specific gene inactivation by insertion of a group II intron, whereas complete coding sequences are deleted by FRAEM and replaced with a marker cassette encoding green fluorescent protein (GFP) and BlaM. Ectopic expression of epitope-tagged gene products has also been employed to study effector localization and function (16–18). Most recently, the expression of enzymatically dead Cas9 was leveraged for conditional knockdown of targeted messages (19).

Organization of genes into polycistronic operons is a common strategy for coordinated gene expression in bacteria. For example, high-resolution RNA sequencing (RNA-seq) analyses have indicated over 1,500 operons in *Escherichia coli* K-12 (20). Consistent with a reductionist genome, many genes in *C. trachomatis* are likely also arranged in operons. Indeed, genes encoding the basal T3S apparatus are arranged within 10 different operons (21). T3SE-specific genes are distributed throughout the genome, and several of the Inc proteins are clearly encoded within operons (22). In addition, the translocated membrane-associated effectors A and B (TmeA and TmeB, respectively) are cotranscribed. The two effectors share the same T3S chaperone (23) and are secreted during invasion (24, 25). FRAEM-mediated mutagenesis revealed an active requirement for TmeA in invasion of cultured cells and in intravaginal infection of mice (26). Importantly, the *tmeA* strain also failed to express *tmeB*. Although the invasion phenotype could be complemented by transexpression of *tmeA*, the loss of *tmeB* complicates the interpretation of functional study results.

The FRAEM and TargeTron techniques require the insertion of fluorescence reporter and/or antibiotic resistance genes to detect and/or select for successful gene disruptions. These insertions have the potential to alter the expression of cotranscribed genes. It would therefore be advantageous to have a mechanism of creating markerless gene deletions to avoid the possibility of polar effects. Bacteriophage Cre recombinase has been used effectively for genome editing of DNA flanked by engineered *loxP* recognition sequences (27). In particular, the Cre-*lox* system has been applied successfully in genetically tractable bacteria (28), including the intracellular pathogen *Coxiella burnetii* (29). Although the obligate intracellular nature and comparatively limited malleability of *Chlamydia* spp. present challenges, the broadly efficacious Cre-*lox* approach represents an attractive possibility for genome editing in *Chlamydia* species.

The goal of the work presented herein was to develop an approach for markerless gene deletion using floxed insertion cassettes and transiently expressed Cre recombinase. We focused on *tmeA* to test whether removal of the reporter cassette from a *tmeA* deletion strain would restore the expression of TmeB. A floxed-cassette *tmeA* mutant
was generated via FRAEM, and transient production of Cre was accomplished by expression with the suicide plasmid pSUmC. The expression of Cre resulted in excision of the \textit{gfp-bla} cassette, and cultivation under nonselective conditions resulted in curing of the suicide plasmid and \textit{cre}. Importantly, the new \textit{tmeA} mutant expressed \textit{TmeB}, and the new \textit{tmeA} mutant retained the previously observed invasion phenotype. Hence, the application of Cre-lox genome editing in \textit{C. trachomatis} can be utilized in combination with FRAEM for markerless gene deletion. This approach provides the field with a novel technique for genetic manipulation of \textit{C. trachomatis}.

**RESULTS**

Excision strategy and \textit{cre} activity. We hypothesized that the lack of TmeB in \textit{C. trachomatis} L2 \textit{tmeA} was due directly to the replacement of \textit{tmeA} with the 2.1-kb \textit{gfp-bla} selection marker. We chose to employ Cre-lox genome editing to investigate whether removal of the cassette could restore \textit{tmeB} expression in the \textit{tmeA} mutant strain. This strategy requires a mutant strain created using a suicide plasmid where a \textit{gfp-bla} selection cassette, flanked by 34-bp \textit{loxP} sites, replaces the target gene. The strain is then transformed with a Cre-encoding plasmid. Expression is maintained until excision of the chromosomal cassette is accomplished, yielding a single \textit{loxP} scar sequence in place of the targeted gene (Fig. 1A). Transient production of Cre in \textit{Chlamydia} spp. was accomplished by expression of \textit{cre} on the suicide plasmid pSUmC. In addition to constitutively expressed Cre, pSU-CRE expresses AadA and mCherry for selection and detection, respectively (Fig. 1B). \textit{E. coli} was used to test the functional integrity of Cre and to confirm the overall efficiency of the system. \textit{E. coli} was cotransformed with pSU-CRE and ploxP-GFP expressing \textit{blaM} on the plasmid backbone and a floxed cassette containing \textit{gfp}. Cotransformation with pSUmC was used as a negative control. We examined 100 plated colonies by direct fluorescence 24 h after transformation. No green bacteria were observed in the presence of pSU-CRE, whereas all colonies were both red and green in the presence of empty pSUmC (Fig. 1C). To

![Figure 1](https://example.com/figure1.png)
confirm excision of the cassette from ploxP-GFP, plasmid DNA was harvested from multiple E. coli isolates, and the cassette locus was PCR amplified using primers annealing to regions flanking the loxP sites. All amplicons from the pSU-CRE-transformed E. coli strains migrated at a size smaller than the pSUmC control, corresponding to loss of the gfp-containing DNA (Fig. 1D). These data indicated that the pSU-CRE-encoded recombinase was functional and that excision was efficient in E. coli.

We next investigated the expression of Cre by chlamydiae. C. trachomatis L2 was transformed with pSU-CRE, and protein samples were harvested from monolayers infected with either wild-type (WT) or C. trachomatis/pSU-CRE for 24 h. Cre was detected via immunoblotting only in the presence of pSU-CRE (Fig. 2A). Although the inclusions formed by pSU-CRE-expressing chlamydiae appeared to be normal (not shown), we wanted to confirm that the expression of Cre had no obvious negative impact on chlamydial fitness. Chlamydia spp. expressing or lacking Cre were used to infect HeLa cells for 24 h. Cultures were then either methanol fixed and stained for inclusion area analysis or disrupted for enumeration of progeny inclusion-forming units (IFU). As expected, there was no significant difference in inclusion areas or production of progeny IFUs. Therefore, Cre is stably expressed in Chlamydia spp. and does not overtly impact chlamydial development.

**Generation of a markerless tmeA mutant.** Our approach to create a markerless tmeA strain began by creating a new tmeA mutant. WT C. trachomatis L2 was transformed with pSUMC-tmeA-lox-gfp-bla, and FRAEM was performed as previously described (15, 30). Isolates were then subjected to cultivation in the presence of rifampin (Rif) to select Rif-resistant chlamydiae, and a clonal strain was derived by limiting dilution. The resulting strain, L2Rrif tmeA-lox-gfp-bla, contained a floxed gfp-bla cassette in place of tmeA and served as the progenitor for genomic editing (Fig. 3A). We were unable to transform this strain with pSU-CRE after several attempts. We therefore leveraged lateral gene transfer (LGT) to mobilize pSU-CRE from WT L2 by coinfection with L2Rrif tmeA-lox-gfp-bla and selecting for red and green inclusions that were resistant to Rif, penicillin G (PenG), and spectinomycin (Spec). Cultures were maintained in the presence of anhydrotetracycline (aTc) to promote the retention of pSU-CRE. Red-only inclusions were observed after one passage in the absence of PenG, indicating successful excision of the gfp-bla cassette. Because this process yielded a mixed population of chlamydiae (lacking or still containing the gfp-bla cassette), Rif- and Spec-resistant red-only Chlamydia spp. were clonally isolated by limiting dilution. Curing of pSU-CRE was then achieved by multiple passages in the absence of Spec and aTc. Phenotypic and PCR analyses indicated that the resulting strain, L2Rrif tmeA-lx, had also been cured of the endogenous plasmid pL2 (not shown). We therefore performed coinfections with WT L2 and L2Rrif tmeA-lx to restore pL2 via LGT to yield L2Rrif tmeA-lx.

![FIG 2 Cre is expressed in Chlamydia spp. and does not impact development. HeLa cells were infected for 24 h with equivalent IFUs of C. trachomatis expressing (+CRE) or lacking (-CRE) pSU-CRE. (A) Whole-culture material was probed in immunoblots with Cre-specific antibodies or anti-Hsp60 as the loading control and visualized via chemiluminescence. (B) Cultures were methanol fixed and stained for inclusion visualization using indirect immunofluorescence. Areas of 50 representative inclusions were measured and plotted individually, with the means and standard deviations shown. (C) Primary cultures were harvested, and progeny Chlamydia spp. were enumerated after secondary passage onto fresh HeLa cells.](https://j.asm.org/content/200/24/e00479-18.full)
Sensitivity to PenG was assayed as an indicator for loss of the gfp-bla cassette (Fig. 3B). Genomes of Chlamydia spp. grown in antibiotic-supplemented media were enumerated over three passages. In contrast to L2 tmeA retaining gfp-bla, WT and tmeA-lx mutant Chlamydia sp. levels dropped below detection by the third passage with PenG. Finally, we confirmed the presence of pL2 via quantitative PCR (qPCR) (Fig. 3C). Levels of pL2 in the tmeA-lx mutant were comparable to the WT, indicating restoration of the endogenous plasmid. Overall, our strategy resulted in the generation of a markerless tmeA deletion strain.

We next focused directly on the tmeA locus to verify that genome editing occurred in accordance with our design. Analysis of genomic DNA via qPCR from the WT, tmeA mutant, or tmeA-lx mutant Chlamydia strains confirmed the absence of tmeA and retention of tmeB (Fig. 4A). In addition, a gfp-specific signal was detected for only the tmeA mutant, and no cre-specific signal was detected for any strain. These data confirmed the complete loss of both the gfp-bla cassette and cre in the tmeA-lx mutant. Excision of the gfp-bla cassette should reduce the locus by ca. 2.1 kb. This was tested by PCR using primers flanking the locus (Fig. 4B). The amplicons from WT and tmeA mutant Chlamydia strains migrated at the expected 1.4 and 2.9 kb, respectively. The tmeA-lx amplicon migrated at ca. 0.5 kb, corresponding to the loss gfp-bla. Finally, we directly sequenced the locus (Fig. 4C). As expected, a single loxP site was detected positioned immediately after the tmeA initiation codon and prior to the stop codon. The intervening sequence leading up to tmeB and the GTG start were not altered from those of the WT. In aggregate, our data indicate the successful generation of a markerless tmeA mutant using Cre-lox.

Excision of the gfp-bla cassette reverses polar disruption of tmeB expression. Having established a successful removal of a floxed gfp-bla cassette to yield L2Rif tmeA-lx, we tested whether or not the goal of alleviating polar effects on tmeB was achieved. RNA and protein analyses were conducted on material extracted from 24-h cultures to examine tmeB-specific products (Fig. 5). When signals were normalized for rpoD levels, approximately similar levels of tmeA- and tmeB-specific amplicons were detected in the WT. As expected, no signal was detected for tmeA and tmeB in the
respective null backgrounds. Levels of tmeB appeared to be reduced in the polar tmeA mutant containing a gfp-bla cassette. However, tmeB was restored to WT levels in the tmeA-lx mutant. Previous work has indicated that ct696 is transcribed independently of tmeA and tmeB (25), and no changes in ct696 message were observed among the tested strains. Western blot data for TmeA and TmeB were consistent with reverse transcription-quantitative PCR (qRT-PCR) results (Fig. 5B). TmeB was absent in lysates from tmeA Chlamydia spp. but was restored in the tmeA-lx mutant. Hence, the remaining loxP scar sequence did not affect TmeB expression. Interestingly, TmeB appeared to be more abundant in the tmeA-lx mutant complemented in trans with tmeA.

We have previously shown that the elimination of tmeA manifests as a defect in invasion efficiency that correlates with a reduction in inclusion numbers in primary and secondary cultures (26). Since this strain also lacked TmeB, we could not formally exclude the possibility that the loss of this effector contributed to the observed phenotypes. To ensure valid phenotypic comparisons, we generated a Rif-resistant WT strain expressing pCompAII. Likewise, a tmeA-lx vector-only mutant was used as an isogenic control for the complementation tmeA-lx ptmeA mutant. HeLa cell cultures were infected with chlamydial strains normalized for particles (Fig. 6, left) or IFUs (Fig. 6, right). Inclusions were enumerated in particle-normalized cultures at 24 h postinfection, whereas IFU-normalized cultures were harvested and passaged onto fresh cells for enumeration of progeny EBs. In both cases, infection was significantly reduced for the tmeA-lx mutant compared to the WT. This deficiency could be reversed by complementation, although restoration of progeny yields was not as robust as those seen in primary cultures. Overall, the data are consistent with our findings that TmeA is important for chlamydial development and that this phenotype is independent of TmeB.

DISCUSSION

An obligate intracellular existence presents obvious barriers to direct genetic manipulation of bacteria. As with Coxiella, Anaplasma, and Ehrlichia spp., the addition of a
The biphasic developmental cycle has further complicated progress in *Chlamydia* spp. (31). The ability to transform *Chlamydia* spp. with a stably maintained shuttle vector, however, has ushered in the ability to inactivate targeted chromosomal genes via insertion with group II introns or complete gene deletion using FRAEM (11). Both of

**FIG 5** Removal of the reporter cassette relieves polar effects on *tmeB*. (A) The presence of transcripts downstream of *tmeA* was determined by reverse transcriptase (RT) quantitative PCR. Total RNA was isolated from at 24 hpi from McCoy cells infected at an MOI of 1 with WT, L2 *tmeA*, L2 *tmeB*, or L2*sup* *tmeA-lx*. Transcripts for *tmeA*, *tmeB*, and *ct696* were detected by qRT-PCR, and signals are presented after normalization to *ropD*. ND, none detected. (B) Equal quantities of whole-culture material from 24-h cultures infected with equal IFUs of WT, L2 *tmeA*, L2*sup* *tmeA-lx*, or L2*sup* *tmeA-lx* ptmeA were probed in immunoblots for TmeA and TmeB. Hsp60 was used as a loading control, and proteins were visualized by chemiluminescence.

**FIG 6** L2*sup* *tmeA-lx* manifests a developmental defect in tissue culture. (A) HeLa cultures were infected in triplicate with equal numbers of *Chlamydia* spp. (A) or IFUs (B) using L2*sup* (WT) + pCompAll or L2*sup* *tmeA-lx* expressing pCompAll (vector) or ptmeA to achieve an approximate MOI of 0.1. Cultures were methanol fixed and stained for chlamydiae (left) or processed for enumeration of progeny EBs (right) at 24 hpi. All inclusions were enumerated by fluorescence staining of chlamydiae in fixed samples, and data for direct and progeny counts are represented as mean ± standard deviation of triplicate samples. A Student t test with Welch's correction was used to address significance (*, *P < 0.02; **, *P < 0.005).
these processes require integration of a selectable marker and/or a fluorescence reporter to recover desired strains due to (i) the numerical confines imposed by the requirement for cell culture and (ii) low-frequency events. These engineered elements have the potential to disrupt the possessivity of RNA polymerase and/or translating ribosomes, thereby impacting de novo synthesis of downstream genes. This is an important issue given the propensity of bacteria to organize genes into polycistronic operons (20). While promoter and operon structures have not been well characterized in Chlamydia spp., transcriptome studies clearly indicate the presence of polycistronic messages. Deep sequencing of the Chlamydia L2b transcriptome was not of sufficient depth to identify all transcription start sites (33), yet at least 246 polycistronic transcripts were detected using a similar approach with the closely related Chlamydia pneumoniae (34). Therefore, overcoming the possibility of polar effects inherent in the application of current gene inactivation technologies in Chlamydia spp. is needed.

Chlamydia trachomatis tmeA and tmeB represent invasion-related T3SEs that are cotranscribed as a bicistronic operon (25). TmeB levels were significantly reduced when tmeA was replaced with a gfp-bla cassette via FRAEM (15), raising the possibility of cassette-dependent polar inactivation. In this study, we have devised an approach that sequentially couples FRAEM-mediated gene deletion with Cre-lox-mediated excision of the resulting selection cassettes. This approach was applied successfully to generate a markerless deletion of tmeA that did not negatively impact tmeB expression. Although the Cre-lox system has been used in Coxiella spp. in a two-step gene deletion strategy, mutagenesis was accomplished with the benefit of an axenic medium, and the resulting deletion strains retained a drug resistance cassette (29). Our work represents the first application of Cre-lox technology for markerless gene deletion in an obligate intracellular bacterium during host cell infection.

Cre recombinase mediates the resolution of bacteriophage P1 dimers through recognition and binding to 34-bp direct repeats termed loxP sites (35). The Cre-lox system has been adapted to successfully engineer a wide diversity of genomes, and this includes the excision of selection markers from bacteria (36). For marker removal, Cre must be present/active subsequent to insertion of the floxed selection cassette and then eliminated from the genome after successful excision. One strategy developed for Mycoplasma spp. relied on conditional expression of Cre using a tet-inducible promoter (37). In this instance, Cre was encoded within the selection cassette such that it was lost concomitantly with the excision event. Importantly, the loxP scar sequence that remains after marker excision has not been observed to exert polar effects on downstream genes (29, 38). Our strategy relies on the expression of Cre in Chlamydia spp. using the conditionally replicating plasmid pSUmC (30). Cre is constitutively expressed from pSU-CRE via a blaM promoter, and the plasmid is maintained via selection with Spec and aTC to induce the expression of pgp6. mCherry is also expressed as a fluorescence marker. Given the labor-intensive requirements for genetically manipulating chlamydiae, our first step was to ensure that the pSU-CRE-encoded recombinase was active in E. coli and adequately expressed in WT Chlamydia trachomatis without interfering with development.

Generation of a markerless deletion mutant began with a Chlamydia strain where tmeA had been replaced with a gfp-bla cassette flanked by loxP sites. pSU-CRE was then mobilized into this strain. We are not certain why the strain could not be transformed with pSU-CRE using the conventional CaCl2 method, but we suspect general low transformation efficiency in Chlamydia to be the culprit. During the course of our broader work, we have found that lateral gene transfer (LGT) can be efficiently leveraged to mobilize engineered plasmids among chlamydial strains. The exchange of genomic DNA via LGT occurs at a frequency of $10^{-3}$ to $10^{-4}$ (39), yet we find that plasmid can be transferred 10- to 100-fold more efficiently (not shown). A spontaneous Rif-resistant strain, L2Rif, tmeA-lx-gfp-bla, was generated to allow selective recovery after coinfection with L2 pSU-CRE. Experiments in E. coli (Fig. 1) were consistent with Cre-mediated excision of the marker cassette being highly efficient. This was important since there was no selective pressure for excision of the cassette in Chlamydia species.
Although there was also no selection to recover markerless chlamydiae, we leveraged the fluorescence reporting aspect of our constructs to monitor excision and recover appropriate strains. Indeed, we could readily visualize a minor population of red-only inclusions after a single passage of L2\textsuperscript{Rif} tmeA-\textsubscript{lx}-gfp-bla/pSU-CRE. Consistent with previous observations (15), selective maintenance of an engineered plasmid in chlamydiae results in the eventual loss of endogenous pL2 during this process. Therefore, we leveraged LGT a second time to restore endogenous pL2 after curing of pSU-CRE. The final strain, L2\textsuperscript{Rif} tmeA-\textsubscript{lx}, was used for further study.

Overall, our data were consistent with the marker cassette exerting a polar effect on tmeB in L2 tmeA that was alleviated by excision of gfp-bla in L2\textsuperscript{Rif} tmeA-\textsubscript{lx}. Both message and protein levels for tmeB were decreased relative to the WT in L2 tmeA, raising the possibility that the presence of gfp-bla interfered with mRNA stability and/or translation. Chlamydial fitness comparisons were made using strains isogenic for Rif, since mutations conferring this resistance can impact fitness in some bacteria (40). Vector-only controls were also used given the contributions of pL2-carried genes in chlamydial infection (41) and observations that ectopic expression of fluorescent proteins can impact fitness in some bacteria (42). Similar to L2 tmeA (15), the absence of tmeA in L2\textsuperscript{Rif} tmeA-\textsubscript{lx} resulted in a decrease in chlamydial infectivity. These data are therefore consistent with the proposed role of tmeA in chlamydial invasion. Although successful complementation indicated that this phenotype was due solely to TmeA, the restoration of TmeB in L2\textsuperscript{Rif} tmeA-\textsubscript{lx} will greatly facilitate further study of TmeA function.

In aggregate, our data provide proof of concept that Cre-lox-mediated recombination is an effective technique for manipulation of the chlamydial genome. Due to limitations imposed by culture and genetic manipulation of Chlamydia spp., this method is necessarily somewhat laborious. It is perhaps most appropriately reserved for instances when operon-localized genes are targeted for inactivation. Other applications are clearly possible in addition to alleviation of polar effects. Alternative antibiotic resistance genes have been used to sequentially engineer a chlamydial strain harboring two inactivated genes (43). Given the limited number of effective antibiotics available for positive selection in Chlamydia spp., the Cre-lox system described herein could be exploited as a mechanism to generate multigene mutant strains. Group II introns have been widely used to disrupt chlamydial genes (11), and Cre-lox has been used to excise group II introns in other bacteria (36). However, the lack of a fluorescence reporter in group II introns would likely complicate strain recovery for Chlamydia species. We propose that Cre-lox is therefore most appropriate for use with FRAEM. Based on the wide variety of manipulations that have been accomplished in other genomes, it also possible that more generalized engineering of the C. trachomatis chromosome can be performed using Cre-lox. Inversions, insertions, and gene deletions may become possible as the chlamydial system becomes more tractable.

MATERIALS AND METHODS

Cell culture and organisms. C. trachomatis serovar L2 (LGV 434) and derivative strains were used in these studies (Table 1). Chlamydiae were routinely maintained in either HeLa 229 epithelial cell monolayers (CCL-1.2; ATCC) or McCoy cell monolayers (CRL-1696; ATCC). All cultures were grown in RPMI 1640 medium containing 2 mM L-glutamine (Life Technologies) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Sigma) at 37°C in an environment with 5% CO\textsubscript{2} and 95% humidified air. All infections were accomplished using density gradient-purified EBs (44) centrifuged onto cell monolayers at 20°C for 1 h at 900 \times g. For transformation and FRAEM protocols, chlamydiae were cultivated in the presence of 600 ng/ml penicillin G (PenG; Sigma), 500 \mu g/ml spectinomycin (Spec; Alfa Aesar), and 1 \mu g/ml cycloheximide (Sigma), in addition to 50 ng/ml anhydrotetracycline (aTc) where appropriate. Rifampin (Rif)-resistant strains were generated as described previously (45) by cultivation for 4 passages in 2.5 ng/ml Rif, followed by 4 passages in 5 ng/ml Rif. Clonal isolates for all final Chlamydia strains were obtained as described by 2 sequential limiting dilution passages in 384 plates (30). Chlamydial fitness was assessed by enumeration of infectious progeny or quantitative assessment of inclusion area as described by Mueller et al. (15). Escherichia coli NEB-10\textregisteredI (New England Biolabs) was utilized for cloning procedures and verification of Cre activity. An E. coli dom dcm deletion mutant (NEB) was used as a host to generate unmethylated plasmid DNA used to transform Chlamydia species. E. coli strains

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were routinely grown at 37°C in Luria-Bertani broth (Amresco) or LB agar plates supplemented with 50 μg/ml carbenicillin (Teknova) or 100 μg/ml spectinomycin, as appropriate.

DNA methods. All PCR-based amplifications for cloning were performed using Q5 high-fidelity DNA polymerase (NEB) from plasmid purified from *E. coli* using the Monarch plasmid miniprep kit (NEB). All primers were custom DNA oligonucleotides from Integrated DNA Technologies (see Table S1 in the supplemental material), and final constructs were confirmed by DNA sequencing of engineered regions. The allelic exchange plasmid pSU-Δctl0063 (Figure 1A), and final constructs were confirmed by DNA sequencing of engineered regions. The allelic exchange plasmid pSU-Δctl0063 (Figure 1A), and final constructs were confirmed by DNA sequencing of engineered regions. The allelic exchange plasmid pSU-Δctl0063 (Figure 1A), and final constructs were confirmed by DNA sequencing of engineered regions. The allelic exchange plasmid pSU-Δctl0063 (Figure 1A), and final constructs were confirmed by DNA sequencing of engineered regions. The allelic exchange plasmid pSU-Δctl0063 (Figure 1A), and final constructs were confirmed by DNA sequencing of engineered regions. The allelic exchange plasmid pSU-Δctl0063 (Figure 1A), and final constructs were confirmed by DNA sequencing of engineered regions.

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<td>pL2&lt;sup&gt;+&lt;/sup&gt;; tmeA&lt;sup&gt;+&lt;/sup&gt; abstr,lox&lt;sup&gt;P&lt;/sup&gt; gfp-bla&lt;sup&gt;+&lt;/sup&gt;; Rif&lt;sup&gt;r&lt;/sup&gt; Pen&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>L2&lt;sub&gt;R&lt;/sub&gt; tmeA-&lt;sub&gt;lx&lt;/sub&gt;</td>
<td>pL2&lt;sup&gt;+&lt;/sup&gt;; tmeA&lt;sup&gt;+&lt;/sup&gt; abstr,lox&lt;sup&gt;P&lt;/sup&gt;; Rif&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>L2&lt;sub&gt;R&lt;/sub&gt;tmeA-&lt;sub&gt;lx&lt;/sub&gt; pCompAll</td>
<td>pL2&lt;sup&gt;+&lt;/sup&gt;; tmeA&lt;sup&gt;+&lt;/sup&gt; abstr,lox&lt;sup&gt;P&lt;/sup&gt; gfp&lt;sup&gt;+&lt;/sup&gt; mCherry&lt;sup&gt;+&lt;/sup&gt;; Rif&lt;sup&gt;r&lt;/sup&gt; Spec&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>L2&lt;sub&gt;R&lt;/sub&gt;tmeA-&lt;sub&gt;lx&lt;/sub&gt;-pTmeA</td>
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<sup>a</sup>pL2<sup>+</sup> and pL2<sup>-</sup> refer to the presence and absence of the endogenous *C. trachomatis* plasmid, respectively. The coding sequences for Pgp1 to -8 are present on the engineered plasmids pCompAll, pSU-CRE, and pTmeA.

**Genetic manipulation of chlamydiae.** Transformation of *Chlamydia* spp. via CaCl<sub>2</sub> and allelic exchange mutagenesis via FRAEM were accomplished as described previously (26). Briefly, 2 μg of unmethylated pSU-CRE or pSU-CmC-tmeA-lox-gfp-bla was used to transform WT *C. trachomatis* L2, and transformants were selected with Spec or Pen, respectively. Generation of the *tmeA-lx* mutant was accomplished by first cultivating pSU-CmC-tmeA-lox-gfp-bla transformants in the absence of aTc for multiple passages, followed by clonal isolation of Pen-resistant GFP-expressing chlamydiae. The deletion of *tmeA* was confirmed by PCR screening with the surroundingt0063-F and surroundingt0063-R primers (Table S1) and direct DNA sequencing. The intermediate strain, L2 *tmeA-lox-gfp-bla*, was cultivated in Rif as described above, and spontaneous Rif-resistant chlamydiae were isolated. pSU-CRE was mobilized into these chlamydiae by lateral gene transfer, L2<sub>R</sub>*tmeA-lox-gfp-bla* chlamydiae were used with WT expressing pSU-CRE to coinfert McCoy cells at a 10:1 ratio. Cultures were maintained for two 48-h passages in the presence of aTc and cycloheximide but in the absence of antibiotic selection. Five serial passages (48 h each) were then performed in the presence of Rif, Spec, aTc, and cycloheximide. Rif- and Spec-resistant chlamydiae expressing mCherry but not GFP were isolated by limiting dilution. Strains were cultivated for five serial passages in Rif and cycloheximide, followed by clonal isolation of Rif-resistant chlamydiae lacking mCherry. The absence of gfp and bla was confirmed by PCR and penicillin sensitivity assays, respectively. The absence of *tmeA* was confirmed as described above via PCR and DNA sequencing. Endogenous plL2 was restored via lateral gene transfer by coinfection with *C. trachomatis* L2 tmeA (15) and L2<sub>R</sub>*tmeA-lx*. The final L2<sub>R</sub>*tmeA-lx* strain was isolated by limiting dilution from cultures exposed to Rif. A spontaneously Rif-resistant WT strain was also isolated and, along with the *tmeA-lx* mutant, was transformed with pCompAll (15) using Spec selection for the production of vector-only controls. Complementation of the *tmeA* mutation was accomplished by transformation of *tmeA-lx* with pTmeA (26).
Microscopy. E. coli colonies were imaged at ×20 using bright-field or epifluorescence microscopy. Chlamydial inclusions were routinely visualized via GFP or mCherry fluorescence in live cultures or via indirect immunofluorescence of methanol-fixed cultures using Hsp60 (Santa Cruz), followed by secondary antibody conjugated to Alexa Fluor 594 (Invitrogen). For quantitative studies, whole-cell fluorescence images were acquired using the Cell Insight CX5 platform (Thermo Scientific) and analyzed using HSC Studio version 6.6 (Thermo Scientific).

Immunoblotting. Whole-culture material was harvested 24 h postinfection (hpi) monolayers infected at a multiplicity of infection (MOI) of 1. Proteins were precipitated in 10%(vol/vol) trichloroacetic acid in phosphate-buffered saline (PBS) and solubilized using 3% Laemmli buffer. Proteins were then resolved by 12%(vol/vol) SDS-PAGE gels and transferred to Immobilon-P membranes (Millipore). Cre recombinase (Cell Signaling), TmeA (24), TmeB (25), or Hsp60-specific antibodies were used to probe the blots. Proteins were visualized by probing with horseradish peroxidase-conjugated secondary antibodies and chemiluminescence detection using Amersham ECL Plus (GE Healthcare UK Limited).

Statistical analysis. All presented data are representative of a minimum of three experiments. Unless otherwise noted, quantitative data were generated from experiments containing triplicate biological samples and duplicate technical replicates. Calculation of standard deviation of the mean and assessment via Student’s t test statistical analyses were performed using Prism 6, version 6.04 (GraphPad Software, Inc., La Jolla, CA).

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at https://doi.org/10.1128/JB.00479-18.

SUPPLEMENTAL FILE 1, PDF file, 0.03 MB.

ACKNOWLEDGMENTS
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21. Hefty PS, Stephens RS. 2007. Chlamydial type III secretion system is


