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RESEARCH ARTICLE

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, chlamydiae 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

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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). $CaCl_2$ 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 disruption. 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

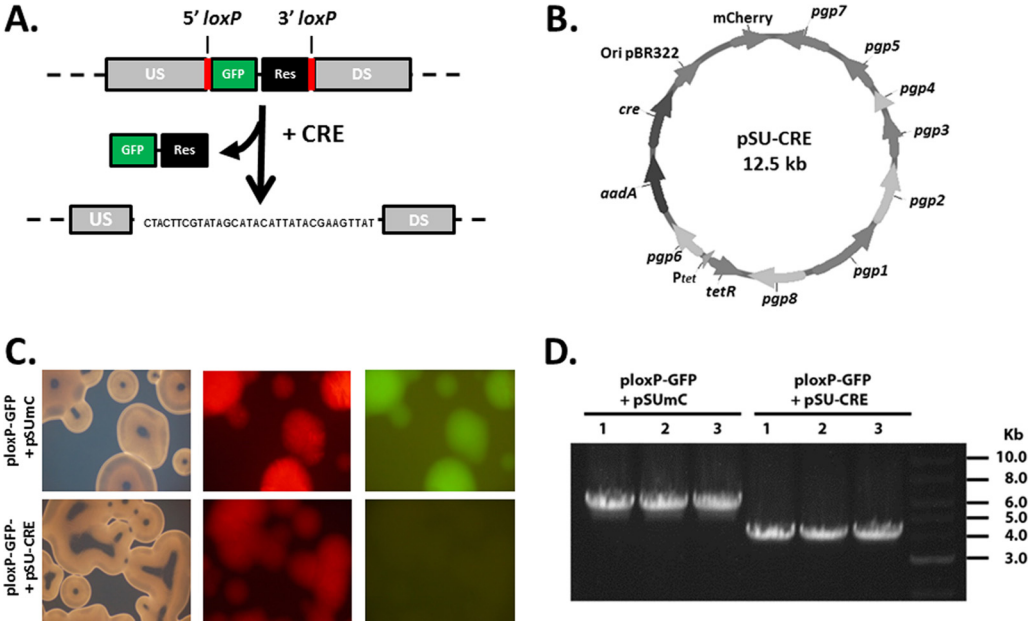

FIG 1 Cre recombinase excises a fluorescence cassette in *E. coli*. (A) Schematic representation of Cre recombinase strategy for use in *Chlamydia* spp. Cre expression is used to excise the GFP and β-lactamase resistance (Res) reporter genes when flanked by upstream (US) and downstream (DS) *loxP* sites, leaving behind a *loxP* scar sequence. The resulting locus is shown and contains one remaining *loxP* site. (B) Schematic of pSU-CRE plasmid for conditional expression of Cre in *C. trachomatis*. (C) *E. coli* colonies expressing ploxP-GFP and lacking (pSUmC) or expressing (pSU-CRE) Cre recombinase. Colonies were imaged with bright-field and fluorescence microscopy. (D) Excision of the reporter cassette was confirmed for three different transformants (1 to 3) by PCR amplification of the locus with primers annealing within the upstream and downstream flanking regions.

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 *gfp-bla* cassette, and cultivation under nonselective conditions resulted in curing of the suicide plasmid and *cre*. Importantly, the new *tmeA* mutant expressed TmeB, and the new *tmeA* mutant retained the previously observed invasion phenotype. Hence, the application of Cre-*lox* genome editing in *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 *C. trachomatis*.

RESULTS

Excision strategy and *cre* activity. We hypothesized that the lack of TmeB in *C. trachomatis* L2 *tmeA* was due directly to the replacement of *tmeA* with the 2.1-kb *gfp-bla* selection marker. We chose to employ Cre-*lox* genome editing to investigate whether removal of the cassette could restore *tmeB* expression in the *tmeA* mutant strain. This strategy requires a mutant strain created using a suicide plasmid where a *gfp-bla* selection cassette, flanked by 34-bp *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 *loxP* scar sequence in place of the targeted gene (Fig. 1A). Transient production of Cre in *Chlamydia* spp. was accomplished by expression of *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). *E. coli* was used to test the functional integrity of Cre and to confirm the overall efficiency of the system. *E. coli* was cotransformed with pSU-CRE and ploxP-GFP expressing *blaM* on the plasmid backbone and a floxed cassette containing *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

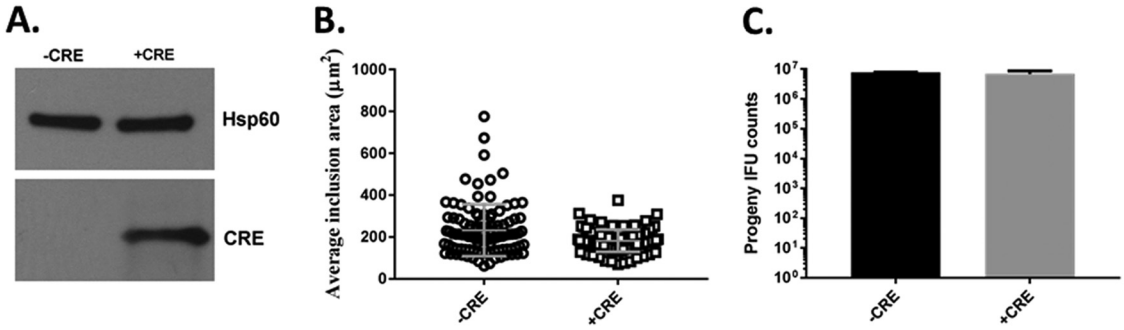

FIG 2 Cre is expressed in *Chlamydia* spp. and does not impact development. HeLa cells were infected for 24 h with equivalent IFUs of *C. trachomatis* expressing (+CRE) or lacking (-CRE) pSU-CRE. (A) Whole-culture material was probed in immunoblots with Cre-specific antibodies or anti-Hsp60 as the loading 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.

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, L2^{Rif} *tmeA-lx-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 L2^{Rif} *tmeA-lx-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, L2R^{Rif} *tmeA-lx*, had also been cured of the endogenous plasmid pL2 (not shown). We therefore performed coinfections with WT L2 and L2R^{Rif} *tmeA-lx* to restore pL2 via LGT to yield L2^{Rif} *tmeA-lx*.

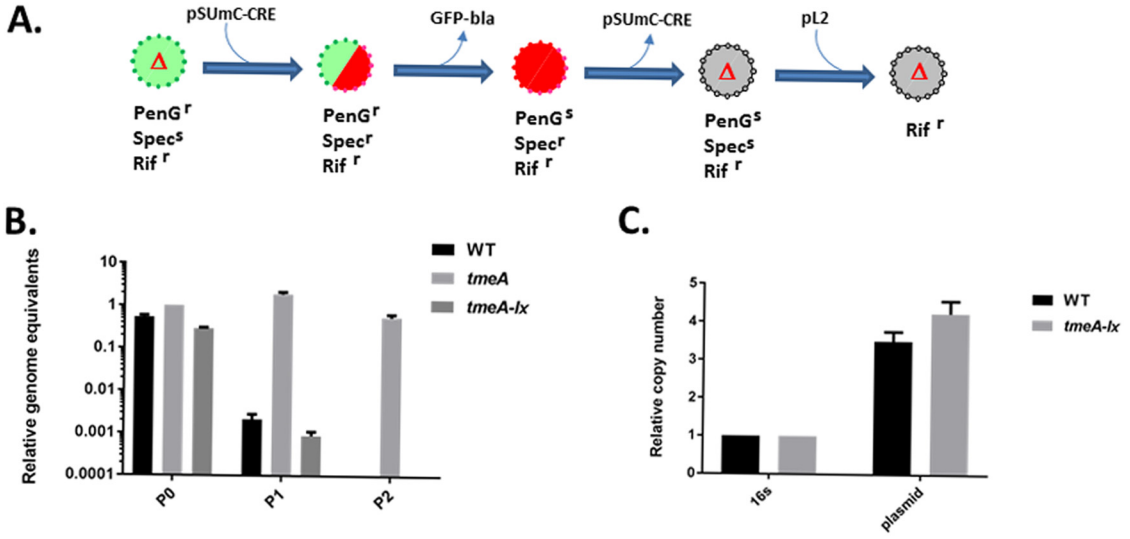

FIG 3 Construction of a markerless *Chlamydia tmeA* mutant. (A) Schematic representation of the strategy used to create a markerless *tmeA* mutant. Each intermediate is depicted with fluorescent qualities (green, GFP$^+$; red, mCherry$^+$; gray, no fluorescence) and antibiotic sensitivities. (B) McCoy cell cultures were infected with equal IFUs of WT, L2 *tmeA*, or L2^{Rif} *tmeA-lx C. trachomatis* and serially passaged every 24 h in medium containing PenG. At each passage, DNA was harvested for quantitative real-time PCR to determine genome equivalents based on chlamydial 16S rRNA. (C) qPCR-based comparison of endogenous pL2 (plasmid) copy number in WT or *tmeA-lx* mutant chlamydiae relative to chlamydial 16S rRNA. DNA was harvested from infected McCoy cells at 24 hpi. PenG^r, penicillin G resistant; PenG^s, penicillin susceptible; Rif^r, rifampin resistant; Spec^s, spectinomycin susceptible; Spec^r, spectinomycin resistant.

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 L2^{Rif} *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

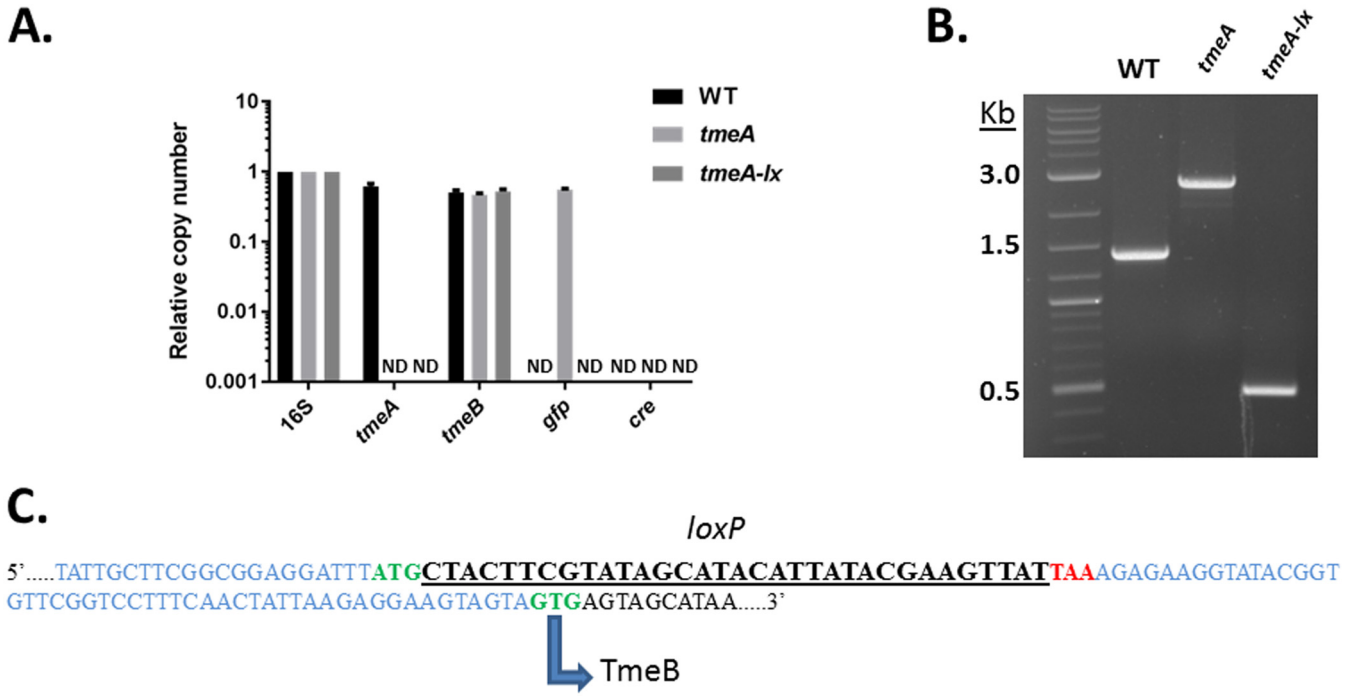

FIG 4 Direct evidence of *gfp-bla* excision. (A) McCoy cells infected with equal IFUs of WT, L2 *tmeA*, or L2^{Rif} *tmeA-lx C. trachomatis* were harvested at 24 hpi, and DNA was extracted for qPCR. Relative copy numbers for *tmeA*, *tmeB*, *gfp*, and *cre* were assessed by signal normalized to chlamydial 16S rRNA. ND, none detected. (B) Excision of the reporter cassette confirmed by PCR by amplifying the *tmeA* locus with primers in the surrounding upstream and downstream regions. Products are shown resolved in a 1.0% agarose gel. (C) The sequenced *tmeAB* locus from L2^{Rif} *tmeA-lx* indicating the remaining *loxP* scar sequence (underlined). Flanking DNA appears in blue, while start codons are in green and the *tmeA* stop is highlighted in red. The noncanonical start codon for TmeB is also depicted.

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 pCompAll. 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

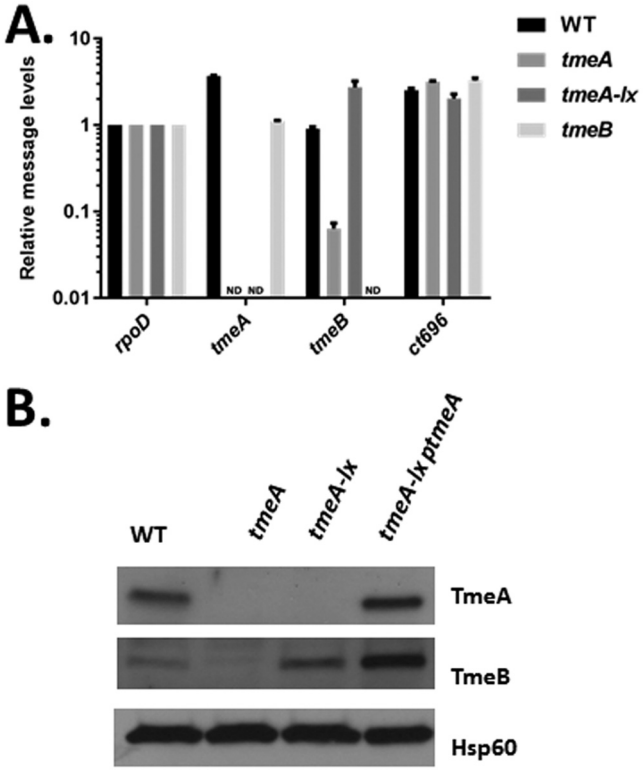

FIG 5 Removal of the reporter cassette relieves polar effects on *tmeB*. (A) The presence of transcripts downstream of *tmeA* was determined by reverse transcriptase (RT) quantitative PCR. Total RNA was isolated from at 24 hpi from McCoy cells infected at an MOI of 1 with WT, L2 *tmeA*, L2 *tmeB*, or L2^{Rif} *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^{Rif} *tmeA-lx*, or L2^{Rif} *tmeA-lx* ptmeA were probed in immunoblots for TmeA and TmeB. Hsp60 was used as a loading control, and proteins were visualized by chemiluminescence.

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

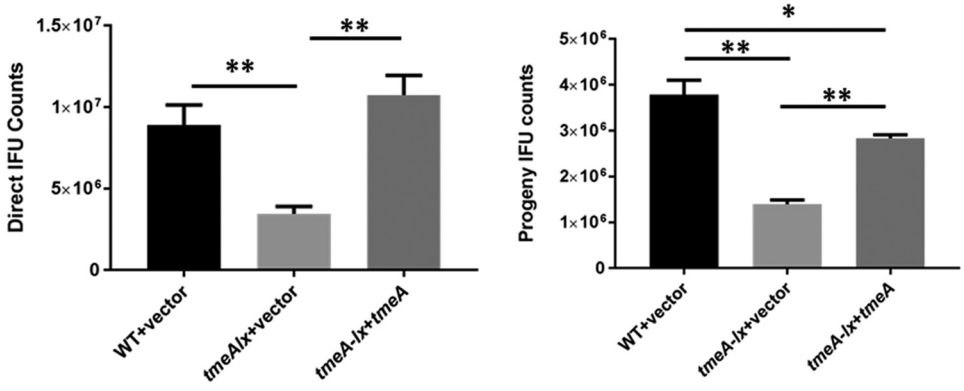

FIG 6 L2^{Rif} *tmeA-lx* manifests a developmental defect in tissue culture. (A) HeLa cultures were infected in triplicate with equal numbers of *Chlamydia* spp. (A) or IFUs (B) using L2^{Rif} (WT) + pCompAll or L2^{Rif} *tmeA-lx* expressing pCompAll (vector) or ptmeA to achieve an approximate MOI of 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. (32), transcriptome studies clearly indicate the presence of polycistronic messages. Deep sequencing of the *C. trachomatis* 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.

C. 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 *C. 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 $CaCl_2$ 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, $L2^{Rif}$ *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^{Rif} *tmeA-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^{Rif} *tmeA-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^{Rif} *tmeA-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^{Rif} *tmeA-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^{Rif} *tmeA-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_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 × *g*. For transformation and FRAEM protocols, chlamydiae were cultivated in the presence of 600 ng/ml penicillin G (PenG; Sigma), 500 μg/ml spectinomycin (Spec; Alfa Aesar), and 1 μg/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 McKuen et al. (26). Primary infections were carried out using particle- or inclusion forming unit (IFU)-normalized chlamydiae, as indicated. Penicillin sensitivity assays were carried out exactly as described by Mueller et al. (15). *Escherichia coli* NEB-10β (New England Biolabs) was utilized for cloning procedures and verification of Cre activity. An *E. coli dam dcm* deletion mutant (NEB) was used as a host to generate unmethylated plasmid DNA used to transform *Chlamydia* species. *E. coli* strains

TABLE 1 *C. trachomatis* strains

Strain designation	Plasmid; relevant genotype; antibiotic resistance[a]	Reference or source
L2 (WT)	pL2$^+$; *tmeA*$^+$	ATCC
L2^{Rif}	pL2$^+$; *tmeA*$^+$; Rif^r	This study
L2R	pL2$^-$; *tmeA*$^+$	47
L2/pSU-CRE	pL2$^+$; *tmeA*$^+$ *cre*$^+$ *mCherry*$^+$; Spec^r	This study
L2^{Rif}/pCompAll	pL2$^-$; *tmeA*$^+$ *gfp*$^+$ *mCherry*; Rif^r Spec^r	This study
L2 *tmeA*	pL2$^+$; *tmeA* absent, *gfp-bla*$^+$; Pen^r	15
L2^{Rif} *tmeA-lx-gfp-bla*	pL2$^+$; *tmeA* absent, *loxP*$^+$ *gfp-bla*$^+$; Rif^r Pen^r	This study
L2R^{Rif} *tmeA-lx*	pL2$^-$; *tmeA* absent, *loxP*$^+$; Rif^r	This study
L2^{Rif} *tmeA-lx*	pL2$^+$; *tmeA* absent, *loxP*$^+$; Rif^r	This study
L2^{Rif}/*tmeA-lx* pCompAll	pL2$^-$; *tmeA* absent, *loxP*$^+$ *gfp*$^+$ *mCherry*$^+$; Rif^r Spec^r	This study
L2^{Rif}/*tmeA-lx* pTmeA	pL2$^-$; *tmeA*$^+$ *loxP*$^+$ *mCherry*$^+$; Rif^r Spec^r	This study
L2 *tmeB*	pL2$^+$; *tmeA* absent, *gfp-bla*$^+$; Pen^r	15

[a]pL2$^+$ and pL2$^-$ 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.

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-Δ*tmeA-lox-gfp-bla* was generated by the addition of *loxP* sites flanking the *bla-gfp* cassette. Flanking *loxP* sites were added via insertion PCR (iPCR) by amplification of the previously described pUC18Δctl0063 (15) using the loxP-blagfp-F and loxP-blagfp-R primers. DNA containing the floxed *gfp-bla* cassette flanked by chlamydial DNA 3 kb up- and downstream of the deleted *tmeA* sequence was amplified with HOMRR@pSUmC-F and HOMRR@pSUmC-R primers. The amplicon was then used in iPCR with pSUmC to yield pSUmC-*tmeA-loxP*. pSU-CRE was constructed first by PCR amplifying the complete CRE coding sequence from pSF-CMV-CRE (Sigma) using primers CRE@pUC18A-F and CRE@pUC18A-R. The amplicon was used in an iPCR reaction to replace *bla* in pUC18A (30) with the CRE-encoding DNA. Cre and upstream *aadA* were then PCR amplified using CRE-aadA@pSUmC-F and CRE-aadA@pSUmC-R and mobilized into pSUmC via iPCR. A bla promoter (P_{bla}) was inserted upstream of *cre* via iPCR amplification of pSU-aadA-CRE with primers loxP-blagfp-F and loxP-blagfp-R. The resulting amplicon was kinased, blunt-end ligated, and transformed into *E. coli* to yield pSU-CRE.

Cre activity in *E. coli* was assessed by cotransformation of *E. coli* with pUC18 containing a floxed *gfp* cassette and pSUmC or pSU-CRE. Transformants were screened by fluorescence and PCR with sense and antisense screening primers (Table S1). PCR screening of chlamydial loci with specific primer sets (Table S1) was accomplished by harvesting whole-culture DNA using 0.5 N NaOH-mediated lysis, as described previously (46). For quantitative PCR, iTaq Universal SYBR green supermix (Bio-Rad) and gene-specific primers (Table S1) were employed. For assessment of gene expression, the Aurum total RNA minikit (Bio-Rad) was used to isolate RNA from McCoy cultures infected at a multiplicity of infection (MOI) of 1. Subsequent generation of cDNA was achieved using the QuantiTect reverse transcription kit (Qiagen), and cDNAs were amplified with iTaq Universal SYBR green supermix (Bio-Rad) and gene-specific primers (Table S1).

Genetic manipulation of chlamydiae. Transformation of *Chlamydia* spp. via $CaCl_2$ and allelic exchange mutagenesis via FRAEM were accomplished as described previously (30). Briefly, 2 μg of unmethylated pSU-CRE or pSUmC-*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 pSUmC-*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 surroundctl0063-F and surroundctl0063-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^{Rif} *tmeA-lox-gfp-bla* chlamydiae were used with WT expressing pSU-CRE to coinfect 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 via PCR and penicillin sensitivity assays, respectively. The absence of *tmeA* was confirmed as described above via PCR and DNA sequencing. Endogenous pL2 was restored via lateral gene transfer by coinfection with *C. trachomatis* L2 *tmeA* (15) and L2R^{Rif} *tmeA-lx*. The final L2^{Rif} *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-well 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 house 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.

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