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A Functional Core of IncA Is Required for *Chlamydia trachomatis* Inclusion Fusion

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ABSTRACT

***Chlamydia trachomatis* is an obligate intracellular pathogen that is the etiological agent of a variety of human diseases, including blinding trachoma and sexually transmitted infections. Chlamydiae replicate within a membrane-bound compartment, termed an inclusion, which they extensively modify by the insertion of type III secreted proteins called Inc proteins. IncA is an inclusion membrane protein that encodes two coiled-coil domains that are homologous to eukaryotic SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment receptor) motifs. Recent biochemical evidence suggests that a functional core, composed of SNARE-like domain 1 (SLD-1) and part of SNARE-like domain 2 (SLD-2), is required for the characteristic homotypic fusion of *C. trachomatis* inclusions in multiply infected cells. To verify the importance of IncA in homotypic fusion in *Chlamydia*, we generated an *incA*::*bla* mutant. Insertional inactivation of *incA* resulted in the formation of nonfusogenic inclusions, a phenotype that was completely rescued by complementation with full-length IncA. Rescue of homotypic inclusion fusion was dependent on the presence of the functional core consisting of SLD-1 and part of SLD-2. Collectively, these results confirm *in vitro* membrane fusion assays identifying functional domains of IncA and expand the genetic tools available for identification of chlamydia with a method for complementation of site-specific mutants.**

IMPORTANCE

***Chlamydia trachomatis* replicates within a parasitophorous vacuole termed an inclusion. The chlamydial inclusions are nonfusogenic with vesicles in the endocytic pathway but, in multiply infected cells, fuse with each other to form a single large inclusion. This homotypic fusion is dependent upon the presence of a chlamydial inclusion membrane-localized protein, IncA. Specificity of membrane fusion in eukaryotic cells is regulated by SNARE (soluble *N*-ethylmaleimide sensitive factor attachment receptor) proteins on the cytosolic face of vesicles and target membranes. IncA contains two SNARE-like domains. Newly developed genetic tools for the complementation of targeted mutants in *C. trachomatis* are used to confirm the minimal requirement of SNARE-like motifs necessary to promote the homotypic fusion of inclusions.**

Chlamydiae are Gram-negative obligate intracellular bacteria that infect both humans and animals. All chlamydiae share a unique biphasic developmental cycle characterized by a metabolically inactive infectious elementary body (EB) and a metabolically active, replicative form termed the reticulate body (RB) (1). EBs bind specific receptors on a susceptible host cell, triggering internalization into a plasma membrane-derived vacuole that is rapidly modified by the bacteria to establish a replicative compartment termed the inclusion (2). Throughout the developmental cycle, the pathogen engages many host organelles, signaling networks, and the cytoskeleton to acquire host lipids, amino acids, and iron, all while suppressing activation of the immune response (3, 4). Starting at about 18 h, a proportion of RBs differentiate back to EBs which accumulate within the inclusion until they are released from the host cell by lysis or extrusion, which occurs between 48 and 72 h postinfection (5).

Chlamydiae encode a type III secretion system that is utilized to secrete bacterial effector proteins. A subset of these secreted effectors are inserted into the inclusion membrane and thus are poised to mediate crucial host-pathogen interactions (6). These inclusion membrane proteins, referred to as Incs, are defined by the presence of a bilobed hydrophobic domain of 40 amino acids (7). The presence of this domain has been used to predict up to 59 Incs in *C. trachomatis* (8–11); the number of Incs varies in other chlamydial species. Generation of specific antibodies (8, 9, 12, 13) and expression of epitope-tagged recombinant Incs in *C. trachomatis* (14, 15) have validated at least 37 of these as bona fide Incs; however, the function of most of these proteins is unknown.

In cells multiply infected with *C. trachomatis*, the inclusions fuse to form a single large vacuole (16, 17). This homotypic vesicle fusion is dependent on the presence of the IncA inclusion membrane protein. Microinjection of antibody to IncA into the cytosol of *C. trachomatis*-infected cells inhibits inclusion fusion (6). Clinical isolates deficient in IncA have been identified and also do not

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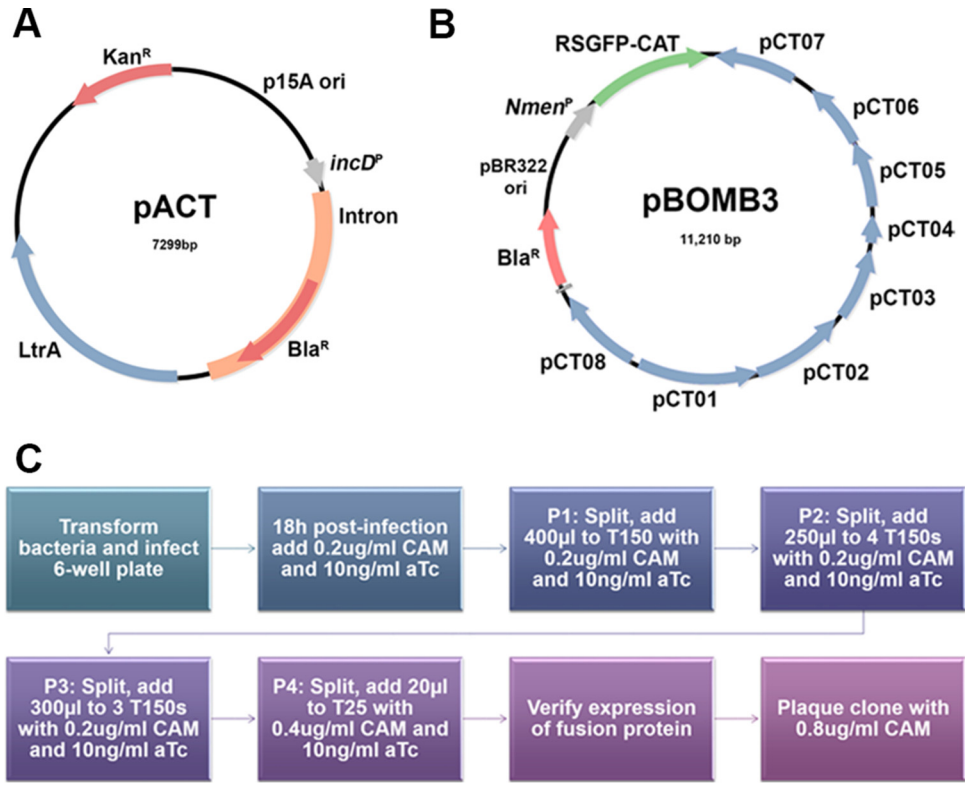

FIG 1 Construction of pACT and pBOMB3 for mutation and complementation in *C. trachomatis*. (A) Map of pACT used for site-specific mutagenesis. (B) Map of pBOMB3 used for complementation. The pBOMB3 vector is similar to the previously reported pBOMB4 except that it contains the *cat* gene fused to the red-shifted green fluorescent protein gene, enabling selection for this vector with chloramphenicol. It also maintains the BamHI and PstI restriction sites within the native L2 plasmid backbone. (C) Flow chart depicting the methods used to transform and select for chloramphenicol (CAM)-resistant, complemented strains.

undergo inclusion fusion (18), supporting the idea of a role of IncA in this fusion event.

Eukaryotic SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment receptor) proteins play an integral role in membrane fusion through the assembly of multimeric complexes that facilitate or inhibit fusion of lipid bilayers (19–21). Given their crucial role in membrane fusion, SNARE proteins represent prime targets for bacterial effector proteins, particularly for those pathogens which occupy parasitophorous vacuoles. IncA, an ~30-kDa inclusion membrane protein, possesses two putative α-helical SNARE-like domains, termed SNARE-like domain-1 (SLD-1) and SNARE-like domain 2 (SLD-2), that functionally mimic eukaryotic SNARE motifs (22–24). IncA is exposed on the cytosolic face of the inclusion membrane (6) and promotes homotypic fusion of inclusions but also inhibits SNARE-mediated membrane fusion, possibly to avoid fusion with endocytic compartments (6, 22–24). Recently, a functional domain, encompassing SLD-1 and part of SLD-2, has been identified (23, 24). However, it has not been determined whether this domain is necessary and sufficient for promoting inclusion fusion in *C. trachomatis* without IncA in a wild-type background (23, 24).

Until recently, a lack of genetic tools significantly hindered our ability to understand the molecular mechanisms of pathogenesis for this important obligate intracellular pathogen. Recent advances, including plasmid transformation (14, 25), generation of random mutants through chemical mutagenesis (26, 27), and adaption of a group II intron-based approach allowing selectable site-specific gene inactivation (28), have facilitated studies aimed at identifying and characterizing chlamydial virulence factors. In the current report, we highlight the importance of IncA in mediating homotypic inclusion fusion using these recently developed genetic approaches. Importantly, we implemented a complementation system that can be used to complement site-specific mutants. Through this system, we demonstrate the necessity of a functional core of IncA for promoting homotypic inclusion fusion in *C. trachomatis*.

MATERIALS AND METHODS

Bacterial and cell culture. *Chlamydia trachomatis* serovar L2 (LGV 434/Bu) was propagated in HeLa 229 cells (American Type Culture Collection) and EBs were density gradient purified as previously described (29). HeLa and Vero CCL-81 cells (ATCC) were grown in RPMI 1640 media (Invitrogen) containing 10% fetal bovine serum (FBS) at 37°C and 5% CO_2.

TargeTron construction. All restriction enzymes and ligases, phosphatases, and DNA polymerases were purchased from New England BioLabs (NEB; Beverly, MA) unless otherwise specified. Oligonucleotides and primers used in this study were purchased from Integrated DNA Technologies (IDT; Skokie, IL) unless otherwise specified. All cloning was performed in *Escherichia coli* DH5α MAX Efficiency competent cells (Life Technologies, Carlsbad, CA).

The Targetron pACDK4-C plasmid was purchased from Sigma-Aldrich (Atlanta, GA) and modified for intron integration in chlamydia (Fig. 1A). The kanamycin–retrotransposition-activated marker cassette for postintegration selection was removed by digestion with MluI. The *bla*

TABLE 1 Primers used in this study[a]

Primer name	Sequence	Use
CT119 24/25 IBS	AAAAAAGCTTATAATTATCCTTAGGCAACGGCTGCGTGCGCCCAGATAGGGTG	CT_119 KO[a]
CT119 24/25 EBS1	CAGATTGTACAAATGTGGTGATAACAGATAAGTCGGCTGCTATAACTTACCTTTCTTTGT	CT_119 KO
CT119 24/25 EBS2	TGAACGCAAGTTTCTAATTTCGATTTTGCCTCGATAGAGGAAAGTGTCT	CT_119 KO
EBS Universal	AATTAGAAACTTGCGTTCAGTAAACACAACTTATAC	CT_119 KO
Tet Promoter SacII F	CCCCGCGGATAATTTTAATTATATCACGGATCC	Complementation
Flag SalI R	GCGCGTCGACCTACTTGTCATCGTCATCCTTGTAGTC	Complementation
pACT Insert F seq	CAGATAAAATATTTCTAGCTAGATTTCAGTGC	Sequencing
pACT Insert R seq	CCAGTTAGTGTTAAGTCTTGGTAAATTCAG	Sequencing
pBOMB Tet F Seq	GGGTTGTTAAACCTTCGATTCCGACC	Sequencing
pBOMB R2 Seq	GCAAAAACAGGAAGGCAAAATGCCGC	Sequencing
CT119 NS NotI F	CCGCGGCCGCTTGATGGACAAAATTAAGAAAATAGC	Sequencing
CT119 NS Flag SalI R	CCGTCGACTTACTTATCGTCGTCATCCTTGTAATCGGAGCTTTTTGTAGAGGGTGAT	Sequencing

[a] KO, knockout.

gene encoding penicillin resistance was PCR amplified from chlamydial plasmid pBOMB4 (14) using primers with 5′ MluI sites. The amplicon was digested with MluI and ligated into pACDK4-C to make pACDP4-C.

The chloramphenicol acetyltransferase resistance cassette (*cat*) located on the pACDK4-C plasmid backbone originally used for selection in *E. coli* was removed. The *cat* gene was removed by whole-plasmid PCR amplification of the pACDP4-C plasmid using primers pACD CATrmv F/R with incorporated 5′ XmaI sites. The primer sequences were specific to the up- and downstream regions immediately flanking *cat* in pACDP4-C, producing a linear DNA product of 6,486 bp. The DNA product was purified and then digested with XmaI. The *aphA1* kanamycin resistance gene, an aminoglycoside 3-phosphotransferase suitable for selection in *E. coli*, was amplified from plasmid pMW1650 using primers pACD Kan Xma F/R containing 5′ XmaI restriction sites. pADP4-C and the purified *aphA1* PCR product were digested with XmaI and ligated to form the pAP4-C kanamycin-resistant plasmid.

The T7 promoter was removed through digestion of pAP4-C with ClaI and HindIII and replaced with a polylinker containing three unique restriction sites for the modularity of promoters used to drive the group II intron. The polylinker contained (from 5′ to 3′) ClaI, DraIII, and HindIII sites and was inserted upstream of a 5′ exon by digestion of pAP4-C with ClaI and HindIII. The polylinker was ligated to the cut, phosphorylated pAP4-C vector to create pAPL-C. The upstream promoter region of chlamydial gene *incD* was amplified from *C. trachomatis* L2 genomic DNA using primers pACD incD-P F/R containing DraIII and HindIII sites incorporated into the forward and reverse primers, respectively. The PCR product was digested with DraIII and HindIII, purified, and then ligated to a DraIII/HindIII-digested pAPL-C vector to create pACT-C.

The intron was retargeted for *C. trachomatis* 434/Bu *incA* using the TargeTron computer algorithm (TargeTronics). Insertion sites with the highest score and the closest proximity to the 5′ ATG start codon were selected. Using the primers listed in Table 1, the intron was retargeted and amplified using a Qiagen core PCR kit (Qiagen). The PCR product was cloned into the BsrGI/HindIII site of pACT, and the ligated plasmid was transformed into methylation-deficient *Escherichia coli* K-12 ER2925 (New England BioLabs). The integrity of all constructs was verified by sequencing.

Complementation vector construction. The pBOMB3 construct was cloned using methods described by Bauler and Hackstadt (14) (Fig. 1B). Briefly, this vector contains regions from the pGFP::SW2 vector constructed by Wang et al. (25) but is modified to contain the entire L2 plasmid backbone and to remove any unnecessary regions, reducing vector size. There are two major differences between the pBOMB4 vector and the pBOMB3 vector. (i) The *cat* gene fused to the red-shifted green fluorescent protein-encoding gene was not removed from the pBOMB3 vector, enabling selection for this vector with chloramphenicol. (ii) the pBOMB3 vector maintains the BamHI and PstI restriction sites within the native L2 plasmid backbone, limiting the selection of enzymes available for use in the multiple-cloning site. To complement the *incA*::*bla* mutant, the tetracycline-inducible promoter and Flag-tagged *incA* were PCR amplified from pBOMB4-Tet-*incA*, pBOMB4-Tet-*incA* core, pBOMB4-Tet-*incA* 1-141, and pBOMB4-Tet-*incA* F/D 1-141 (23) using the primers listed in Table 1. All PCR products were cloned into the SacII/SalI site of pBOMB3 and transformed into *E. coli* K-12 ER2925.

Isolation of an *incA*::*bla* mutant. *C. trachomatis* serovar L2 was transformed with pACT-*incA* as previously described (14, 15, 28). Plasmid DNA was transformed into *C. trachomatis* serovar L2 (LGV 434/Bu) density gradient-purified EBs using $CaCl_2$ buffer (10 mM Tris [pH 7.5], 50 mM $CaCl_2$). At 18 h postinfection, 0.1 U/ml penicillin G was added and cultures were incubated an additional 24 h. Following three passages, transformants were plaque cloned in Vero cells and individual plaques were picked and expanded. Disruption of *incA* was verified using PCR and sequencing of genomic DNA isolated from plaque-purified bacteria using a Qiagen blood and tissue kit (Qiagen).

Complementation of *C. trachomatis incA*::*bla* mutant. To adapt a system of complementation for site-specific mutants, we focused on the *incA*::*bla* mutant because of the readily observed phenotype associated with disruption of IncA. A plaque-purified *incA*::*bla* mutant was expanded and EBs were density gradient purified as previously described (29). The transformation was conducted as previously described (30) with modifications outlined below and shown in Fig. 1C.

(i) A total of 10^7 purified EBs were mixed with 100 μl $CaCl_2$ buffer and 3 μg DNA. Following a 30-min incubation at room temperature, 4.5 ml RPMI medium with 10% FBS was added, and 2 ml of the mixture was added to two wells of a HeLa monolayer in a 6-well plate. Cultures were centrifuged for 30 min at 2,400 rpm and were subsequently incubated at 37°C with 5% CO_2.

(ii) At 18 h postinfection, 0.2 μg/ml chloramphenicol was added, IncA expression was induced with 10 ng/ml anhydrous tetracycline (aTc), and cultures were incubated an additional 24 h.

(iii) At 40 to 48 h postinfection, medium was removed and host cells were lysed in 1 ml sterile water/well. Host cell debris were pelleted for 5 min at 1,500 rpm, and 400 μl of supernatant was applied to a new HeLa monolayer in a T150 flask with RPMI 1640 plus 10% fetal bovine serum (FBS), 1 μg/ml cycloheximide, 0.2 μg/ml chloramphenicol, and 10 ng/ml aTc (passage 1 [P1]).

(iv) Following 40 to 48 h of incubation, cultures were split by lysing in 1 ml sterile water (total) and 250 μl was applied to 4 new HeLa monolayers (P2) in T150 flasks with RPMI 1640 plus 10% fetal bovine serum (FBS), 1 μg/ml cycloheximide, 0.2 μg/ml chloramphenicol, and 10 ng/ml aTc.

(v) At 40 to 48 h postinfection, the cultures were again split as described above and 300 μl of supernatant was applied to 3 new HeLa monolayers in a T150 flask containing RPMI 1640 plus 10% fetal bovine serum (FBS), 1 μg/ml cycloheximide, 0.2 μg/ml chloramphenicol, and 10 ng/ml aTc (P3).

(vi) At 40 to 48 h postinfection, the cultures were again split as de-

scribed above and 20 μl of supernatant was applied to new HeLa monolayers in a T25 flask containing RPMI 1640 plus 10% fetal bovine serum (FBS), 1 μg/ml cycloheximide, 0.4 μg/ml chloramphenicol, and 10 ng/ml aTc (T3). The remaining supernatant was pelleted at 12,000 rpm for 20 min, and the pellet was resuspended in sucrose phosphate glycerol (SPG) buffer and stored at −80°C.

(vii) At 40 to 48 h postinfection, host cells were lysed as conducted above. The entire supernatant was pelleted at 12,000 rpm for 20 min and the pellet was resuspended in SPG buffer. Expression of the Flag-tagged fusion protein was verified using immunofluorescence microscopy.

(viii) Complemented mutants, expressing the fusion protein, were plaque cloned in Vero cells under selection with 0.8 μg/ml chloramphenicol, and individual plaques were picked and expanded.

Immunofluorescence. HeLa cells were seeded at 10^5/ml onto glass coverslips and after 24 h were infected at an multiplicity of infection (MOI) of 1. IncA expression was induced with 10 ng/ml of aTc, and, 24 h postinfection, cells were fixed with 100% methanol and blocked using 1% BSA and DNA was stained with DAPI (4′,6-diamidino-2-phenylindole; Invitrogen). EBs were stained with anti-Momp antisera, and IncA was visualized using either anti-IncA antisera or an anti-Flag antibody (Sigma). Primary antibodies were observed by staining with goat anti-rabbit IgG–DyLight 594 and goat anti-mouse IgG–DyLight 488 (Jackson Laboratories) secondary antibodies. Images were captured on a Nikon Eclipse 80i fluorescence microscope and analyzed using Nikon Elements software. Data are representative of results of at least two independent experiments performed with at least 100 infected cells per experiment.

Growth curve analysis. HeLa cells were infected on ice with an MOI of 1 with each bacterial strain, and expression of IncA was induced with 10 ng/ml aTc. After 30 min on ice, cultures were shifted to 37°C to stimulate bacterial uptake. At 0 h, 4 h, 12 h, 24 h, 36 h, and 48 h, postinfected cells were lysed in water and supernatants were applied to fresh HeLa monolayers to enumerate inclusion-forming units (IFUs).

Western blotting. HeLa cells were infected with an MOI of 5, and expression of IncA was induced with 10 ng/ml aTc. At 24 h postinfection, cells were lysed in 1% SDS and analyzed by immunoblotting.

Southern blotting. A 5-μg volume of genomic DNA from *incA*::*bla* L2 and wild-type *C. trachomatis* L2 was digested to completion with HindIII. As a positive control, 750 ng of purified pACT vector was also linearized by digestion with HindIII. Digested DNA was resolved in a 5-mm-thick 1% agarose gel, stained with ethidium bromide, and photographed in a UV cabinet. DNA ladder standards were marked, and the resolved DNA was transferred to a Hybond-N (GE Healthcare; Pittsburgh, PA) nylon membrane via capillary transfer with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (pH 7.0) overnight. Following transfer, the blot was UV cross-linked using a Stratalinker 1800 instrument (Stratagene, Los Angeles, CA) at 70,000 J/cm^2. The Hybond-N membrane was dried and hybridized with an 866-bp probe specific to *bla*, an intron-specific gene encoding ß lactamase.

The probe was labeled with dCTP (α-^{32}P) (PerkinElmer, Shelton, CT) (3,000 Ci/mmol, 10 mCi/ml) using a DecaPrime II labeling kit (Life Technologies) and then purified using Illustra microspin G-25 Sephadex columns (GE Healthcare, Pittsburgh, PA).

The membrane was prehybridized for 2 h at 42°C using 20 ml of hybridization solution (50% formamide, 6× SSC, 5× Denhardt's solution, 0.5% SDS) with 50 μg/ml denatured salmon sperm DNA. Following prehybridization, 20 ml of hybridization solution was combined with 10 μl of probe and the blot was hybridized with probe overnight at 42°C with shaking. The nylon membrane was washed, dried, and exposed to CL-Xposure film (Thermo Scientific; Atlanta, GA) for 24 h.

RESULTS

Generation of *C. trachomatis incA*::*bla* mutant. To disrupt IncA, the TargeTron pACD4K vector was modified for use in *C. trachomatis* (Fig. 1A) essentially as previously described (28), except the strong *incD* promoter was used to drive the intron in place of the CTL0665 promoter and kanamycin resistance was used for selection in *E. coli*. The intron was retargeted to *incA* using primer sequences obtained from TargeTronics. Primers that resulted in an insertion nearest the 5′ ATG and had the highest score were selected for retargeting. The resulting construct, pACT-*incA*24, was transformed into *C. trachomatis* serovar L2 (LGV 434/Bu), and transformants were expanded under conditions of penicillin G selection for four passages and were subsequently plaque purified. As previously described (28), viable inclusions were readily observed in passage 2 (P2) and nonfusogenic inclusions were apparent in P4 when the MOI exceeded 1. To verify disruption of IncA, genomic DNA was isolated from wild-type *C. trachomatis* serovar L2 (LGV 434/Bu) and from the L2 *incA*::*bla* mutant. PCR was employed to amplify *incA* using gene-specific primers. Sanger sequencing of *incA*::*bla* verified insertion of the intron in the antisense orientation relative to *incA* (Fig. 2A), as predicted by the TargeTronics algorithm. Agarose gel electrophoresis of the PCR product confirmed insertion of the group II intron as indicated by the 1.7-kB shift in the mutant band relative to the wild-type results (Fig. 2B). To confirm that loss of IncA expression was due to premature termination, immunofluorescence analysis of wild-type- and L2 *incA*::*bla* mutant-infected cells was conducted. As shown in Fig. 2C, IncA membrane staining was clearly evident on wild-type inclusions but was absent from *incA*::*bla* mutant-infected cells. Furthermore, nonfusogenic inclusions were present only in L2 *incA*::*bla* mutant-infected cells. No significant differences between the strains in growth rate were noted (Fig. 2D). Southern blotting was used to confirm a single intron insertion site (Fig. 2E). Collectively, these results indicate successful disruption of IncA.

Generation of a complementation vector. The pBOMB3 vector was constructed to facilitate complementation of genetic knockout mutants. It contains a chloramphenicol acetyltransferase gene (*cat*) to enable selection of the vector using chloramphenicol, an antibiotic that is not currently used for medical treatment of *C. trachomatis* infection and that has been shown to function for selection of plasmid maintenance in *Chlamydia* (30). The pBOMB3 vector contains the entire backbone of the native L2 plasmid; therefore, genetic knockout mutants complemented with this vector do not lack any additional genetic information formerly available on the native plasmid. Finally, the pBOMB3 vector encodes a red-shifted green fluorescent protein to visualize complemented organisms via immunofluorescence (Fig. 1B).

Complementation of *incA*::*bla* mutant rescues inclusion fusion. To verify that IncA is required for homotypic fusion of *C. trachomatis* inclusions, we expressed Flag-tagged IncA from a tetracycline-inducible promoter using the pBOMB3 vector. The resulting construct was transformed into infectious L2 *incA*::*bla* EBs that had been purified by Renografin density gradient centrifugation. Transformants were passaged and selected for by the use of 0.2 μg/ml chloramphenicol as outlined in Fig. 1C and as described in Materials and Methods. As shown in Fig. 3, induction of IncA expression by the addition of 10 ng/ml aTc resulted in IncA expression and localization to the inclusion membrane as evidenced by staining with anti-IncA (Fig. 3A) or anti-Flag (Fig. 3B) antibodies. In the absence of aTc induction, IncA expression and rescue of inclusion fusion were not observed in cells infected with the complemented strain. No growth difference was noted between the strains (Fig. 3C). Taken together, these results reinforce the idea of

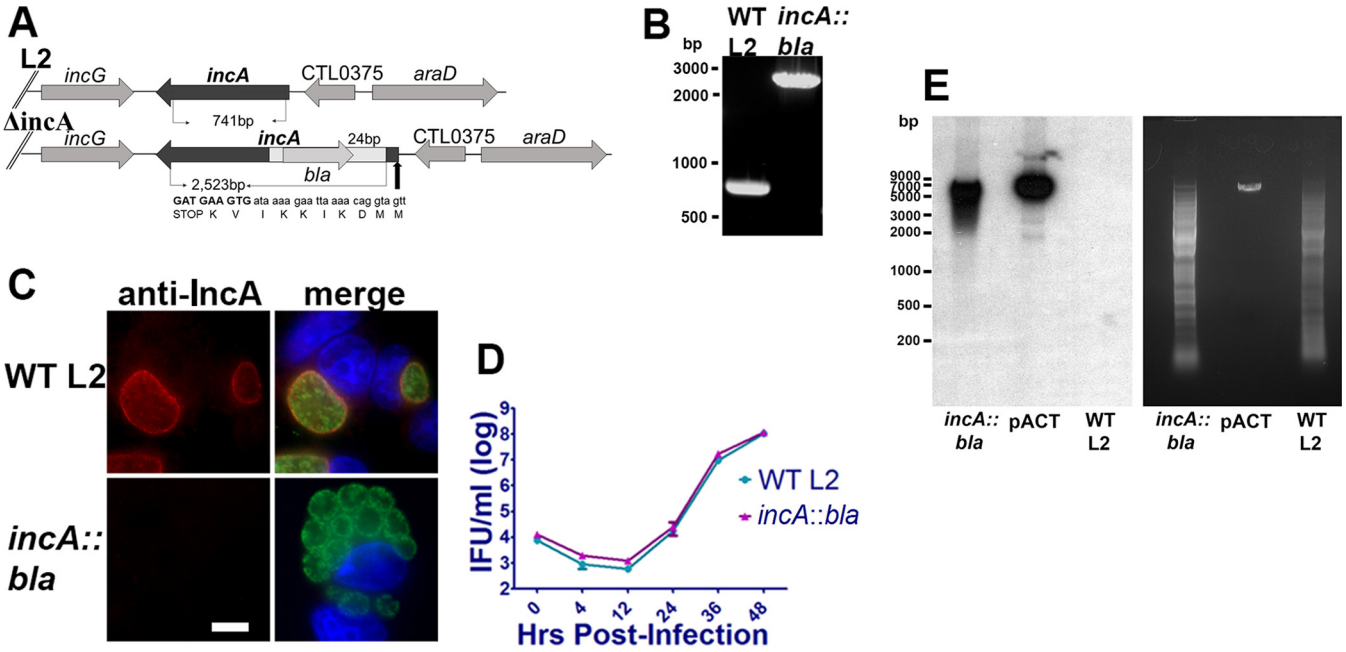

FIG 2 Generation of an *incA*::*bla* mutant. PCR was conducted on genomic DNA isolated from wild-type and *incA*::*bla C. trachomatis* L2. (A) Sanger sequencing of isolated DNA using gene-specific primers was conducted to verify orientation and insertion of the intron. The site of premature termination relative to the start site of *C. trachomatis* 434/Bu *incA* (YP_001654458) is shown. (B) PCR products were separated on a 1% agarose gel, and DNA was visualized using ethidium bromide staining. WT, wild type. (C) Immunofluorescence analysis was conducted on HeLa cells infected with an MOI of 1, and, after 24 h, cells were fixed with methanol and probed with anti-Momp (green) and anti-IncA (red) primary antibodies. Nuclei were stained using DAPI (blue). Data are representative of results of 3 independent experiments performed with at least 100 infected cells observed per experiment. Bar = 10 μm. (D) HeLa cells infected with wild-type L2 or *incA*::*bla* L2 were incubated for 0 h, 4 h, 12 h, 24 h, 36 h, or 48 h. Next, cells were lysed in water and replated on fresh HeLa monolayers to enumerate IFUs. Data are representative of results of 2 independent experiments. (E) Southern blot analysis of *incA*::*bla* L2, plasmid pACT containing *bla*, and parental *C. trachomatis* L2 (WT L2) using *bla* as a probe. A single insertion site is seen in *incA*::*bla* L2.

the importance of IncA in homotypic fusion and establish a system for complementing site-specific mutants in *C. trachomatis*.

Homotypic fusion of inclusions requires a functional core of IncA. The ability to transform and overexpress Incs in wild-type chlamydiae has enabled experiments that assess the function of IncA during infection (23); however, the ability to generate site-specific mutants now allows more-direct evaluation of the function of IncA SNARE-like motifs in mediating inclusion fusion in the absence of a potentially confounding background of wild-type native IncA. IncA is composed of a bilobed hydrophobic domain (amino acids [aa] 34 to 82) and two SNARE-like domains termed SLD-1 (aa 107 to 145) and SLD-2 (aa 210 to 273) (Fig. 4A). Recent studies indicate that inclusion fusion is dependent on the presence of a functional core consisting of SLD-1 and part of SLD-2. To verify that inclusion fusion requires these domains, we expressed mutant IncA and full-length Flag-tagged IncA in the L2 *incA*::*bla* mutant under the control of a Tet promoter. HeLa cells were infected with *C. trachomatis* transformed with the various IncA constructs (Fig. 4A), and, 24 h postinfection, cells were stained using anti-IncA (Fig. 4B) or anti-Flag (Fig. 4C) antibodies. IncA staining at the inclusion membrane was evident for cells infected with *C. trachomatis* expressing either full-length IncA or the IncA core. Cells infected with the *incA*::*bla* mutant expressing IncA SLD-1 alone (pBOMB4-tet-incA 1-141) or an SLD-null construct (pBOMB4-tet-incA F/D 1-141) did not exhibit characteristic IncA staining. The IncA antibody used in this study is specific for the C terminus of IncA, confirming that the latter two strains lack the C terminus. Immunofluorescent staining of the recombinant chlamydia confirmed expression of each of the constructs and appropriate localization into the inclusion membrane of the full-length, core, and SLD1 constructs. The SLD-null (F/D 1-141) construct was not translocated to the inclusion membrane but appeared to be retained in association with the bacteria. Importantly, expression of the IncA core resulted in the formation of a single inclusion, comparably to wild-type *C. trachomatis* or cells complemented with full-length IncA. Multiple, nonfusogenic inclusions were observed in bacteria complemented with IncA SLD-1 or the SLD-null construct. To confirm the expression levels of the various constructs, immunoblotting was performed. As shown in Fig. 4C, expression levels of the induced constructs were equivalent as assessed by anti-Flag staining. Notably, expression levels of IncA under conditions of aTc induction were greater than that of IncA from the wild-type parental strain, as IncA was not expressed to levels detectable by anti-IncA in the parental L2 strain. The IncA antibody was produced against a domain near the C terminus of the protein and thus is not observed in the truncated versions of the protein. To better assess the fusogenicity of these inclusions, we scored cells infected with an MOI of 1 for the presence of multiple inclusions (more than 2 per cell). As shown in Fig. 4D, expression of the IncA core significantly rescued inclusion fusion, whereas mutants complemented with SLD-1 or the SLD-null construct exhibited defects comparable to those seen with the *incA*::*bla* mutation alone, with over half of the infected cells possessing multiple inclusions. Although complementation of the *incA*::*bla* mutant with either full-length IncA or the IncA core significantly restores

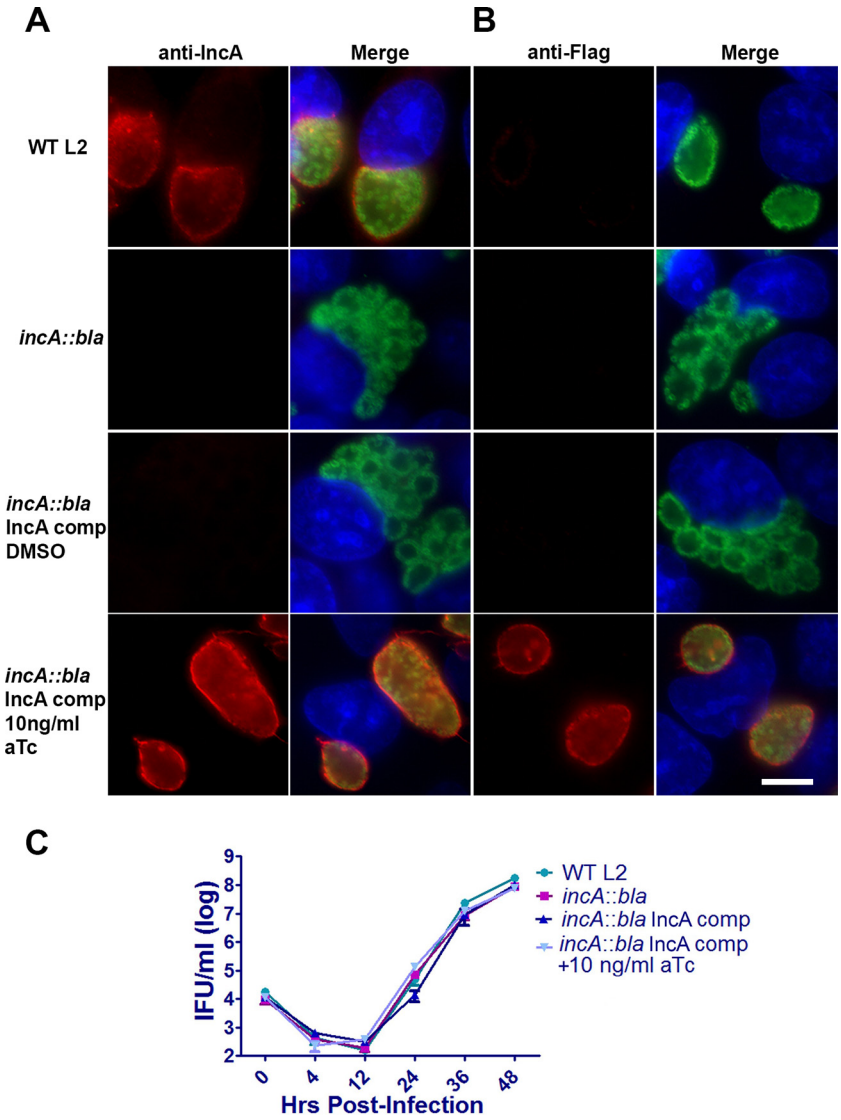

FIG 3 Complementation of *incA*::*bla* L2. (A and B) HeLa cells were infected at an MOI of 1 with wild-type L2 or *incA*::*bla* L2, and, after 24 h, cells were fixed with methanol and probed with (A) anti-IncA (red) and anti-Momp (green) or (B) anti-Flag (red) and anti-Momp (green) antibodies. Nuclei were visualized using DAPI (blue). Data are representative of results of 3 independent experiments performed with at least 100 infected cells per experiment. Bar = 10 μm. (C) For growth comparisons, HeLa cells were infected at an MOI of 1 with wild-type L2 or *incA*::*bla* L2. Infected cells were incubated for 0 h, 4 h, 12 h, 24 h, 36 h, or 48 h, at which point, cells were lysed in water and replated on fresh HeLa monolayers to enumerate IFUs. Data are representative of results of 2 independent experiments.

inclusion fusion, the level of inclusion fusion for the IncA core was not equivalent to that seen with the parental L2 strain ($P < 0.01$). The failure of full-length or core IncA constructs to fully restore fusion to wild-type levels may be due to the abnormally high levels of IncA in the induced recombinant strains. Importantly, the differences in homotypic fusion were not due to differences in the replication rates, as all strains replicated with similar kinetics (Fig. 4E). Collectively, our results indicate that the functional core composed of SLD-1 and part of SLD-2 is necessary to promote IncA-mediated inclusion fusion.

DISCUSSION

Until recently, the genetic intractability of chlamydiae significantly hindered our ability to understand this important pathogen. In the past decade, great strides have been made in applying modern molecular biology techniques to the study of chlamydiae. In 2011, Wang et al. (25) described a system for plasmid transformation of *C. trachomatis* EBs using calcium chloride and penicillin selection. Since that seminal study, the genetic toolbox available for use in *C. trachomatis* studies has been expanded to include expression of a variety of fluorescent tags (14, 31), epitope tags (14), inducible promoters (14), and alternative selectable markers (30, 32). Additionally, dendrimers have been used to silence gene expression (33, 34) and a library of random mutants has been generated using chemical mutagenesis (26, 27). Recently, a mobile group II intron was adapted for use in *C. trachomatis* that allows selectable insertional inactivation of target genes (28) and this technology has been extended to insertionally inactivate multiple

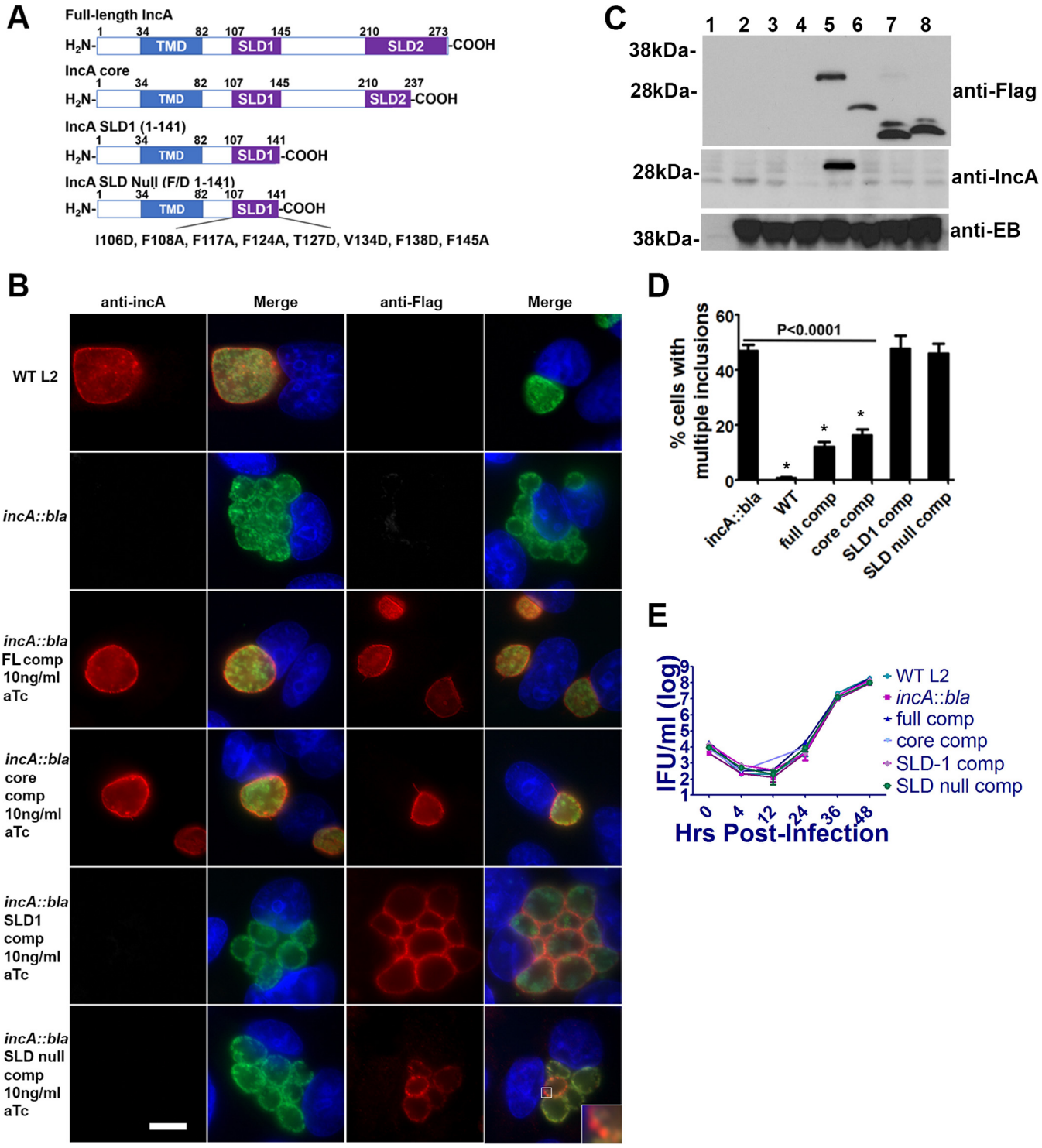

FIG 4 IncA-mediated homotypic fusion requires a functional core composed of SLD-1 and part of SLD-2. (A) IncA is composed of a 34-amino-acid N terminus, a bilobed transmembrane domain (amino acids 35 to 82), SLD-1 (amino acids 107 to 145), and SLD-2 (amino acids 210 to 273). (B) For immunofluorescence analysis, HeLa cells were infected at an MOI of 1 with each strain. At 24 h postinfection, cells were fixed with methanol and probed with anti-IncA (red) and anti-Momp (green) or anti-Flag (red) and anti-Momp (green) antibodies. Nuclei were visualized using DAPI (blue). The inset shows higher magnification of anti-Flag staining with the chlamydia expressing IncA SLD-null. Shown are wild-type L2 (WT L2); *incA*::*bla* L2; *incA*::*bla* L2 IncA full-length complemented (FL comp) induced; *incA*::*bla* L2 core complemented induced; *incA*::*bla* L2 SLD1 (1-141) complemented induced; and *incA*::*bla* L2 SLD null (F/D 1-141) complemented induced. Data are representative of results of 3 independent experiments performed with at least 100 infected cells per experiment. Bar = 10 μm. (C) HeLa cells, infected at an MOI of 2.5 for 24 h and subjected to immunoblotting using anti-Flag, anti-IncA, or anti-EB sera. The 40-kDa band representing Momp is shown. Samples are as follows: lane 1, uninfected cells; lane 2, wild-type L2; lane 3, *incA*::*bla* L2; lane 4, *incA*::*bla* L2 IncA full-length complemented uninduced; lane 5, *incA*::*bla* L2 IncA full-length complemented induced; lane 6, *incA*::*bla* L2 core complemented induced; lane 7, *incA*::*bla* L2 SLD1 (1-141) complemented induced; lane 8, *incA*::*bla* L2 SLD null (F/D 1-141) complemented induced. (D) Quantification of multiple inclusions (more than 2 per cell) was conducted by counting at least 200 infected cells in duplicate. Asterisks indicate differences from *incA*::*bla* L2 at a *P* level of <0.0001. Data are representative of results of 3 independent experiments. (E) Growth curve analyses were conducted with HeLa cells infected at an MOI of 1 with each strain. After 0 h, 4 h, 12 h, 24 h, 36 h, or 48 h, cells were lysed in water and replated on fresh HeLa monolayers to enumerate IFUs.

genes (32). While complementation has been demonstrated for random chemical mutants, the ability to complement specific mutants harboring a selectable marker has been lacking. Here we adapted a chlamydial shuttle vector for complementation of mutants and used this system to verify that a functional core of IncA is required to mediate homotypic inclusion fusion.

To establish a method for complementation of *C. trachomatis* site-specific mutants, we focused on *incA* because of the readily observable phenotype associated with loss of *incA*. Naturally occurring IncA^{-} mutants from clinical samples (18) and a *incA*::*bla* mutant generated by site-specific mutagenesis (35) exhibit a nonfusogenic phenotype. IncA is located in close proximity to other *inc* genes (*incDEFG*) but is regulated independently (13), suggesting that this phenotype is most likely not due to a polar effect. Here we used a modified chlamydial shuttle vector to express full-length Flag-tagged IncA under the control of an aTc-inducible promoter. Inducible expression of IncA rescued IncA localization to the inclusion membrane and homotypic fusion, confirming that IncA is necessary to promote the homotypic fusion of inclusions in *C. trachomatis*.

Previously, we showed that overexpression of recombinant, full-length IncA or IncA core in wild-type *C. trachomatis* has minimal effect on the characteristic homotypic fusion of the inclusions. In contrast, overexpression of either SLD-1 or SLD-2 acted in a dominant-negative fashion to block homotypic fusion with a concomitant increase in the percentage of cells exhibiting multiple, nonfused inclusions (23). Here, we used a group II intron mutant of IncA to evaluate IncA function in the absence of a background of wild-type IncA. While either wild-type IncA-flag or IncA core-flag substantially complemented homotypic fusion of the *incA*::*bla* mutant, the presence of IncA SLD-1 or the IncA SLD-null construct was insufficient to rescue homotypic fusion. Full-length IncA-flag, IncA-core, or IncA SLD-1 was localized appropriately to the inclusion membrane; however, the SLD-null construct was not observed on the inclusion membrane but appeared to remain associated with the bacteria. The phenylalanine/aspartic acid mutations introduced into the SLD-null IncA mutant (pBOMB4-tet-incA F/D 1-141) disrupted its alpha-helical structure, which was necessary to impair its function (21). This loss of secondary structure may account for the absence of secretion and its retention within the bacteria.

In addition to promoting homotypic fusion of *C. trachomatis* inclusions, wild-type IncA and IncA core also inhibit SNARE-mediated membrane fusion (23). The *C. trachomatis* L2 *incA*::*bla* mutant grew at a normal rate regardless of the presence or absence of IncA constructs that did or did not restore fusogenicity of the inclusion. The chlamydial inclusion is well known not to be fusogenic with endosomal/lysosomal compartments (36–39), and this avoidance of interactions with the endocytic pathway is evident quite early in infection (40), occurring before expression of IncA, which is not expressed until about 10 to 12 h in *C. trachomatis* L2 (9). It appears that IncA is not essential for avoidance of fusion with endocytic compartments, at least *in vitro*, and that there may be redundant mechanisms to avoid host defense mechanisms.

The number of molecular genetic tools for experimental manipulation of chlamydiae is increasing rapidly, and new tools promise to continue to strengthen the study of chlamydial pathogenesis. A robust means to complement mutants using a second, selectable marker such as chloramphenicol resistance adds a valuable tool for confirming the function of genes in chlamydia. As an example of how rapidly the field of chlamydia genetics is expanding, a means for allelic exchange by homologous recombination under conditions of beta-lactamase selection was recently described and with it a means for complementation using a separate plasmid expressing spectinomycin resistance (41). The availability of multiple selectable markers can only help accelerate the development of chlamydial genetic systems.

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