Expression and Targeting of Secreted Proteins from *Chlamydia trachomatis*

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Chlamydia trachomatis is an obligate intracellular pathogen that replicates in a vacuole termed the inclusion. Many of the interactions of chlamydiae with the host cell are dependent upon bacterial protein synthesis and presumably exposure of these proteins to the cytosol. Because of the dearth of genetic tools for chlamydiae, previous studies examining secreted proteins required the use of heterologous bacterial systems. Recent advances in genetic manipulation of chlamydia now allow for transformation of the bacteria with plasmids. We describe here a shuttle vector system, pBOMB4, that permits expression of recombinant proteins under constitutive or conditional promoter control. We show that the inclusion membrane protein IncD is secreted in a type III-dependent manner from *Yersinia pseudotuberculosis* and also secreted from *C. trachomatis* in infected cells where it localizes appropriately to the inclusion membrane. IncD truncated of the first 30 amino acids containing the secretion signal is no longer secreted and is retained by the bacteria. Cytosolic exposure of secreted proteins can be confirmed by using CyaA, GSK, or microinjection assays. A protein predicted to be retained within the bacteria, NrdB is indeed localized to the chlamydia. In addition, we have shown that the chlamydial effector protein, CPAF, which is secreted into the host cell cytosol by a Sec-dependent pathway, also accesses the cytosol when expressed from this system. These assays should prove useful to assess the secretion of other chlamydial proteins that are potentially exposed to the cytosol of the host cell.

Chlamydiae are medically significant Gram-negative pathogens of human and veterinary importance. *Chlamydia trachomatis* is a major cause of human morbidity. The species is comprised of over 15 serologically defined variants, or serovars, associated with distinct tissue tropisms and disease states. Serovars A to C are the most common cause of preventable blindness worldwide (1). Serovars D to K are the leading cause of bacterial sexually transmitted disease in the developed world. Serovars L1, L2, and L3 are the etiologic agents of a more systemic disease, also sexually transmitted, called lymphogranuloma venereum (LGV) (2, 3). Other species affecting humans include *Chlamydia pneumoniae*, a common cause of community-acquired pneumonia (4), and *Chlamydia psittaci*, a rare but serious zoonotic disease (5, 6).

Chlamydiae exhibit a biphasic developmental cycle that involves morphologically and physiologically distinct intracellular and extracellular forms (7, 8). The extracellular form, called the elementary body (EB), is the infectious form of the life cycle and thus adapted for extracellular survival. The intracellular, replicative form is known as a reticulate body (RB).

Upon endocytosis, the EB is contained within a plasma membrane derived vesicle which is rapidly modified by the bacteria to establish a replicative niche termed the inclusion (9, 10). The chlamydiae remain within the inclusion for the duration of their intracellular development. The chlamydial inclusion is a unique vacuole that interacts with multiple cellular pathways, organelles, and cytoskeletal components to create a suitable replication niche (11–14). As obligate intracellular parasites, chlamydiae interact extensively with the host cell to ensure availability of nutrients, biosynthetic precursors, and avoidance of host protective responses. Chlamydiae encode at least two pathways known to secrete proteins such that they are exposed to the cytosol. The chlamydial proteasome-like activity factor (CPAF) (15) is secreted by a Sec-dependent or type II secretion pathway (16), where it is further transported to the cytosol by unknown mechanisms. Chlamydiae

also encode a type III secretion system (T3SS) (17–19), a mechanism used by pathogenic bacteria to translocate bacterial effector proteins across host membranes (20). Among these secreted effectors are a number of chlamydial proteins, called inclusion membrane proteins (Incs), which are incorporated into the inclusion membrane such that they are exposed to the cytosol (21) and therefore positioned to potentially regulate interactions with the host cell. Over 50 putative inclusion membrane proteins have been predicted in *C. trachomatis* based upon a characteristic bilobed hydrophobic domain of approximately 40 amino acids (22–26).

Due to its obligate intracellular lifestyle, genetic manipulation of chlamydiae has been a challenge in the field. Recently, a method of plasmid transformation of *C. trachomatis* allowing for the expression of exogenous genetic material has been described (27). Here, we describe a shuttle vector system to express secreted effector proteins tagged with various reporters from *C. trachomatis* and use this system to investigate the ability of *C. trachomatis* to secrete effector proteins into the inclusion membrane and cytosol of host cells during an infection.

MATERIALS AND METHODS

Organisms and cell culture. *C. trachomatis* serovar L2 (LGV 434/Bu) was propagated in HeLa 229 cells (American Type Culture Collection CCL-2.1) cultured in RPMI 1640 medium (Invitrogen) containing 10% fetal bovine

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FIG 1 pBOMB4 cloning vector for *C. trachomatis*. Maps of the pBOMB4 vectors. Open reading frames from the *C. trachomatis* L2 434/Bu vector are labeled ppg1-ppg8. A multiple cloning site containing BamHI, SacII, NotI, NheI, PstI, AgeI, KpnI, and SalI sites was constructed and inserted between the L2 plasmid and β -lactamase gene. The β -lactamase gene, pUC19 origin of replication, and GFP gene were amplified from pGFP-SW2. The pBOMB4R vector is identical to pBOMB4 except that it contains the rpoB promoter (indicated in black). Both pBOMB4 and pBOMB4R have mCherry versions constructed by replacing the GFP gene with an mCherry gene (indicated by gray arrows). The pBOMB4-Tet-mCherry vector contains a tetracycline-inducible promoter and mCherry gene inserted into the BamHI/NotI and NotI/PstI restriction sites of pBOMB4, respectively (indicated by black arrows).

serum (FBS; HyClone) at 37°C and 5% CO₂. Infectious EBs were purified using a Renografin (Braco Diagnostics) density gradient as described previously (28). Chlamydial titers were determined as described previously (29). Progeny EBs were quantified at various time points postinfection by lysing infected cells in distilled water and replating them in triplicate onto fresh HeLa cell monolayers. At 24 h postinfection, monolayers were fixed and stained with a rabbit anti-EB antisera, followed by an anti-rabbit secondary antibody (Jackson ImmunoResearch). Inclusions were counted in 20 fields per sample using a Nikon Eclipse 80i fluorescence microscope, and the numbers of infectious progeny were calculated.

Plasmid construction. The pBOMB4 vector was constructed using GeneArt Seamless cloning (Invitrogen). Primers (Integrated DNA Technologies) used in the construction can be found in Table S1 in the supplemental material. All PCR was performed using the Phusion polymerase (NEB). The plasmid from *C. trachomatis* L2/434Bu was amplified in two parts, from ppg7 to a region in ppg2, and from ppg2 to ppg8. A new multiple cloning site (MCS) containing BamHI, SacII, NotI, NheI, PstI, AgeI, KpnI, and SalI was synthesized as an oligonucleotide and added to the 3'-end of the L2 vector during amplification of that fragment. The β -lactamase gene and promoter and origin of replication were amplified from pGFP:SW2, as was the *Neisseria meningitidis* promoter and GFP-CAT gene. These five segments were assembled using a GeneArt Seamless cloning kit (Invitrogen). The rpoB promoter, was amplified from L2/434Bu genomic DNA, and an overlap-PCR was performed to synthesize a DNA segment containing the second half of the L2 plasmid and the rpoB promoter using DNA from each PCR product as the template. The CAT gene was removed using GeneArt homologous recombination by amplifying the pBOMB4 vector using primers corresponding to the 5' and 3' ends of the CAT gene, which also contained homologous sequences, such that the intervening CAT gene was deleted. The following primer pairs were used: pBOMB.Cat deletion F and L2 overlap R; L2 overlap F and L2 plasmid.R or pBOMB.rpoB.R; pBOMB.Bla.F and pBOMB.Cat deletion.R. A similar approach was used to remove the green fluorescent protein (GFP) gene and replace it with the mCherry gene, which was amplified from the

pMCherry-C1 vector (Clontech). Within the native L2 plasmid there are two restriction endonuclease sites, BamHI and PstI, which are also in the MCS; these native sites were mutated using site-directed mutagenesis to remove them. The resulting vectors were named pBOMB4 and pBOMB4R (contains the rpoB promoter). The tetracycline promoter and CyaA gene were amplified from the pJB-Kan-TetRPA-cyaA vector (30). The tetracycline promoter and mCherry gene were introduced into the MCS of pBOMB4 using BamHI/NotI and NotI/PstI, respectively (NEB), resulting in pBOMB4-Tet-mCherry. All chlamydial genes were amplified from genomic DNA using gene-specific primers with restriction endonuclease sites incorporated into the 5' end of the primers. Primers used are listed in Table S1 in the supplemental material. The 3 $\times$ -Flag tag was amplified from p3xFLAG-CMV-14 (Sigma). The glycogen synthase kinase (GSK) tag was synthesized using oligonucleotide primers. The Flag, GSK and CyaA tags were added to the ends of the target genes via overlap-PCR.

Real-time quantitative PCR. Density gradient-purified EBs were boiled in 20 mM dithiothreitol (DTT) for 10 min to obtain DNA. Primers designed to the *hctA* gene were used for genomic DNA enumeration. For the L2 plasmid, primers were designed across the ppg7-ppg8 junction, which is separated in the pBOMB4 vector by the β -lactamase gene, origin of replication, and GFP gene. For quantitation of the pBOMB4 vector, primers were designed to the GFP gene. Real-time quantitative PCR was performed using Power SYBR green PCR master mix on a 7900HT Fast real-time PCR system (Applied Biosystems). Copy number was calculated as the fold change in $\Delta\Delta C_T$ relative to genomic DNA.

Chlamydial transformation. Plasmid DNA was prepared from a methylation deficient strain K-12 ER2925 *Escherichia coli* (NEB). The following transformation protocol was adapted from procedures developed by Wang et al. (27), Song et al. (31), and Agaisse and Derre (32).

Passage 0 (P0). A total of 10⁸ *C. trachomatis* L2 434/Bu density gradient purified EBs were incubated with 6 μ g of DNA and 200 μ l of CaCl₂ buffer (10 mM Tris [pH 7.5], 50 mM CaCl₂) for 30 min at room temperature. After 30 min, antibiotic-free RPMI 1640 medium was added, and

the bacteria were plated into three wells of HeLa cell monolayers in six-well plates. The cultures were centrifuged at 2,400 rpm in a Jouan CR412 centrifuge for 30 min, followed by incubation at 37°C in 5% CO₂.

Passage 1 (P1). At 42 to 48 h postinfection, wells were scraped into sterile distilled water to lyse host cells. Cellular debris was pelleted at 1,500 rpm for 5 min. The supernatant was used to infect a fresh monolayer of HeLa cells in a T150 flask with RPMI 1640 plus 10% fetal bovine serum (FBS), 1 µg of cycloheximide (Sigma)/ml, and 0.1 U of penicillin G (Sigma)/ml.

Passage 2 (P2). At 42 to 48 h postinfection, flasks were scraped and lysed in water, and cellular debris was pelleted as described above. The supernatant was used to infect a fresh monolayer of HeLa cells in a T25 flask with RPMI 1640 plus 10% FBS and 1 µg of cycloheximide/ml and 0.1 U of penicillin G/ml.

Passage 3 (P3). At 72 h postinfection, flasks were scraped and lysed in water. Cellular debris was pelleted, and the supernatant was used to infect a new monolayer of HeLa cells in a T25 flask with RPMI 1640 plus 10% FBS and 1 µg of cycloheximide/ml and 0.1 U of penicillin G/ml. Viable inclusions were visible as early as 24 h postinfection. Transformed chlamydiae were plaque cloned in Vero cells (ATCC CCL-81). Briefly, Vero cells were infected with appropriate dilutions of chlamydia transformants and incubated with a 5% agarose/RPMI overlay containing 1 µg of cycloheximide (Sigma)/ml and 1 U of penicillin G/ml, until plaques developed. Individual plaques were picked and expanded.

Cyclic AMP (cAMP) assay. The cAMP-Glo assay (Promega) was performed according to the manufacturer's protocols. Briefly, HeLa cells were plated in 96-well plates and infected with *C. trachomatis* at a multiplicity of infection (MOI) of 1 for 30 min at 37°C. RPMI 1640 without serum, containing 500 µM isobutyl-1-methylxanthine (Sigma), and 100 µM 4-(3-butoxy-4-methoxy-benzyl) imidazolidone (Sigma) was added, and cells were incubated for 24 h at 37°C in 5% CO₂. Cells were lysed in cAMP-Glo lysis buffer for 15 min at room temperature (RT), and cAMP-Glo detection solution was then added and incubated at room temperature for 20 min, followed by Kinase-Glo reagent for 10 min at room temperature. Luminescence was measured using a Synergy 4 plate reader (BioTek) with a 1-s integration time.

Antibodies. HeLa cells infected at an MOI of 1 were harvested at 24 h postinfection in 2× SDS-PAGE lysis buffer for immunoblotting or fixed in 4% paraformaldehyde for indirect immunofluorescence. The following antibodies were used: anti-EB antisera, anti-Flag (Sigma), anti-CyaA (Santa Cruz Biotechnologies), anti-GSK-3B-tag (Cell Signaling), anti-phospho-GSK-3B (Cell Signaling), peroxidase-conjugated donkey anti-mouse IgG, peroxidase-conjugated donkey anti-rabbit IgG, DyLight 594-goat anti-rabbit, and DyLight 594-goat anti-mouse (Jackson ImmunoResearch). DAPI (4',6'-diamidino-2-phenylindole; Invitrogen) was used at a concentration of 0.5 µg/ml.

Microinjection. Microinjections were performed as previously described (21, 33). Briefly, *C. trachomatis*-infected cells were injected at 3 h postinfection using a Micromanipulator 5171 and a Transjector 5426 Plus (Eppendorf, Hamburg, Germany). Anti-Flag antibody was used at 10 µg/ml in microinjection buffer (48 mM K₂HPO₄, 14 mM NaH₂HPO₄, 4.5 mM KH₂PO₄ [pH 7.2]). Injections were conducted at room temperature, and the delivery pressure and injection duration for most experiments was 1.4 lb/in² and 0.5 s, respectively. Cells were fixed 24 h postinfection and stained with secondary antibodies.

Yersinia T3SS. *C. trachomatis* genes encoding IncD, IncC, and NrdB were fused to neomycin phosphotransferase and cloned into the pFLAG-CTC vector (Sigma) (34). The constructs were transformed into wild-type (pIB102) and ΔyjcS (pIB68) *Yersinia pseudotuberculosis* YPIII. *Y. pseudotuberculosis* was cultured at 26°C for 2 h in the presence of 5 mM calcium or absence of calcium (20 mM MgCl₂ and 5 mM EGTA) to inhibit or promote type III secretion, respectively. Cultures were shifted to 37°C, and IPTG (0.01 mM) was added at the time of the temperature shift to induce expression of the chlamydial proteins. Cultures were harvested after 4 h of growth at 37°C and separated into cell pellet or supernatant

FIG 2 Characterization of *C. trachomatis* L2 transformed with the pBOMB4 vector. (A) HeLa cells were infected with *C. trachomatis* L2 (●) or one of the following transformed strains: pBOMB4R-IncD-Flag (○), pBOMB4R-IncD-CyaA (▲), or pBOMB4R (■). At various time points postinfection, cells were lysed in water and replated on fresh monolayers to enumerate the IFU (means ± the standard errors of the mean [SEM]; *n* = 3). (B) Density gradient-purified EBs were lysed in 20 mM DTT. Quantitative PCR was performed using primers specific to gDNA, the L2 plasmid, or the pBOMB4 plasmid. Copy number was determined relative to genomic DNA. Shown are the means ± the SEM (*n* = 3).

fractions. Samples were analyzed by immunoblotting with anti-Flag M2 antibodies to detect the presence or absence of each chlamydial protein. Samples were also probed with antibodies directed against the *Y. pseudotuberculosis* type III secretion substrate YopN to determine whether type III secretion was functioning normally in each sample (35).

Nucleotide sequence accession numbers. Sequences of the pBOMB4 plasmids have been deposited in GenBank under accession numbers KF790906, KF790907, KF790908, KF790909, and KF790910.

RESULTS

Construction of the vector. We modified the pGFP:SW2 plasmid to develop a shuttle vector that might be more readily manipulated to express genes of interest in *C. trachomatis*. The pGFP:SW2 plasmid contains the *C. trachomatis* SW2 plasmid as a backbone (27). The pGFP:SW2 vector was constructed by piecing several vectors together using traditional cloning techniques (27), resulting in a vector with restriction endonuclease sites scattered throughout and no multiple cloning site (MCS), limiting the use of restriction enzymes for further cloning. The pBOMB4 vector (Fig. 1) was constructed to remove many of these sites from throughout the vector and to insert a MCS for expression of target genes under the control of constitutive or inducible promoters. The endogenous plasmid from the L2/434Bu strain was amplified and assembled with the β-lactamase gene, pUC origin of replication, the RSGFP gene from pSW2:GFP, and an MCS designed in an oligonucleotide using GeneArt Seamless cloning. An mCherry variant of the pBOMB4 vector was also constructed by replacing the RSGFP gene with the mCherry gene (pBOMB4-MCI). Genes

FIG 3 Tetracycline-inducible promoter driven mCherry expression in *C. trachomatis*. (A) HeLa cells were infected with *C. trachomatis* L2 transformed with pBOMB4-Tet-mCherry for 8 h, at which time various amounts of anhydrotetracycline hydrochloride (aTC) were added to induce the expression of mCherry. All mCherry (red) images were taken at the same exposure. Green images indicate the expression of the GFP gene on the plasmid. (B) HeLa cells were infected as described above. At 24 h postinfection, 50 ng of aTC/ml was added to examine the induction of mCherry over time. Bar, 10 μm.

can be expressed using an *rpoB* promoter (pBOMB4R) upstream of the MCS or a tetracycline-inducible promoter (pBOMB4-Tet) which was cloned into the MCS. The resulting pBOMB4 shuttle vectors are ~1 kb smaller than pGFP:SW2 and can easily be manipulated to express genes of interest.

Characterization of *C. trachomatis* L2 transformed with the pBOMB4 vector. To ensure that the *C. trachomatis* carrying the pBOMB4R plasmid replicated similarly to wild-type chlamydia, we performed a one-step growth curve analysis (Fig. 2A). *C. trachomatis* transformed with either pBOMB4R, pBOMB4R containing an IncD-Flag fusion, or pBOMB4R with an IncD-CyaA fusion, grew at similar rates compared to wild-type bacteria. This indicates that transformed chlamydia, which are constitutively expressing a gene of interest in the pBOMB4R vector, are not negatively affected in their ability to replicate. We also wanted to determine whether the endogenous L2 plasmid was retained after transformation and penicillin selection. Quantitative PCR analysis was performed with primers that amplify genomic DNA, the endogenous L2 plasmid, or the pBOMB4R vector. As shown in Fig. 2B, only the endogenous L2 plasmid is detected in the untransformed *C. trachomatis*, while in the transformed *C. trachomatis* only the pBOMB4R vector is detected, indicating that the transformed bacteria lose the original L2 plasmid after transformation with the pBOMB4R vector.

Characterization of a Tet-inducible promoter in chlamydia. *C. trachomatis* expresses genes throughout its developmental cycle in a sequential pattern of at least three temporal classes, early, midcycle, and late (36, 37). The potential for toxicity of constitutive expression of genes either out of sequence or in abnormal abundance is a concern. The ability to regulate gene expression is thus essential for studies examining the pathogenesis of chlamydia-

FIG 4 IncD is secreted in a type III secretion system-dependent manner in *Yersinia pseudotuberculosis*. *Y. pseudotuberculosis* YPIII pIB102 (WT) or YPIII pIB68 (yscS) expressing IncD, IncD-ΔSS, IncC, NrdB, or vector-only controls were grown in brain heart infusion broth in the presence (+) or absence (-) of 5 mM Ca²⁺. Cultures were incubated at 26°C, after 2 h protein was induced with 0.01 mM IPTG, and cultures were shifted to 37°C for an additional 4 h. Cultures were fractionated into pelleted cells (P) or supernatant (S or Sup). Samples were resolved on an SDS-PAGE gel and immunoblotted with α-Flag M2 (A) or α-YopN (B).

ial infection. We assessed the use of the tetracycline-inducible (tet) promoter in pBOMB4-Tet-mCherry, for expression of proteins by *C. trachomatis* during the infection of host cells. In samples treated with anhydrotetracycline at concentrations of ≥400 ng/ml for 24 h, *C. trachomatis* inclusions were visibly smaller in size than untreated samples, suggesting that higher concentrations of anhydrotetracycline are inhibitory to chlamydial growth (data not shown). Similar sensitivity to anhydrotetracycline was recently described by Wickstrum et al. (38). Figure 3A depicts induction of mCherry under the control of the tet promoter with as little as 10 ng of anhydrotetracycline hydrochloride/ml (Fig. 3A). In separate experiments, Flag-tagged fusion proteins were expressed in the presence of as little as 0.5 ng of anhydrotetracycline /ml (data not shown), a concentration ~100-fold less than the MIC for tetracycline (39). Fluorescent mCherry was visibly detected within 2 h of induction (Fig. 3B). These results indicate that the tetracycline-inducible promoter is extremely sensitive in chlamydia and provides a tool for the rapid induction of genes of interest.

IncD is secreted in a type III secretion system-dependent manner in *Yersinia pseudotuberculosis*. Previous characterization of chlamydia T3SS substrates had to be performed in heterologous systems. *Yersinia pseudotuberculosis*, *Shigella flexneri*, and *Salmonella enterica* serovar Typhimurium had each been used to demonstrate type III secretion of chlamydial effector proteins (17, 34, 40, 41). Heterologous secretion in conjunction with actual localization of the putative effector outside the inclusion had been

FIG 5 Secretion of recombinant products from *C. trachomatis*. HeLa cell monolayers were infected with *C. trachomatis* L2 transformed with pBOMB4-Tet-IncD-Flag, pBOMB4-Tet-IncD-ΔSS-Flag, pBOMB4-Tet-NrdB-Flag, or pBOMB4-Tet-CPAF-Flag for 24 h before fixation with 4% paraformaldehyde for immunofluorescence (A) or lysed in 2× SDS-PAGE buffer for immunoblotting (B). (A) For immunofluorescence, samples were probed with anti-IncD or α-Flag antibody, followed by a secondary antibody of Alexa 594 (red) and counterstained for DNA with DAPI. Bar, 10 μm. (B) The immunoblot was probed with anti-Flag antibody, followed by an HRP-conjugated secondary antibody. The calculated molecular masses of the flag-tagged fusion proteins were as follows: IncD (19.7 kDa), IncD-ΔSS (16.3 kDa), NrdB (43.2 kDa), and CPAF (70.7 kDa). Two apparent proteolytic fragments of CPAF are also visible.

considered evidence for type III secretion. In one case, translocation of a chlamydial T3S effector protein by *Y. pseudotuberculosis* across the plasma membrane of host cells was demonstrated (42). To confirm that the inclusion membrane protein IncD can be secreted via a heterologous T3SS, we expressed IncD fused to the carrier protein neomycin phosphotransferase (Npt) in wild-type *Y. pseudotuberculosis* or a T3SS-deficient strain, Δ*yscS* *Y. pseudotuberculosis* (Fig. 4). We included, as a negative control, a cytosolic chlamydial protein, NrdB. IncC, which has previously been shown to be secreted in a T3SS-dependent manner from *Y. pseudotuberculosis* was included as a positive control (17). These strains were grown in the presence or absence of calcium to inhibit or induce the T3SS, respectively. Protein expression was induced by the addition of IPTG, and the expression of the T3S apparatus was induced by a temperature shift from 26 to 37°C. The supernatants and pellets were collected to determine whether the tagged proteins were secreted into the supernatant. No secreted product was detected in the Npt empty vector, in the NrdB-expressing negative-control samples, or from the T3SS-deficient strains, indicating that the cells were not lysing and releasing protein into the supernatant. However, the positive control, IncC, was secreted by the wild-type *Y. pseudotuberculosis* but not the T3SS-deficient Δ*yscS* strain. IncD-Flag was secreted from the wild-type *Y. pseudotuberculosis* transformed with the IncD construct but not from the IncD construct which had the first 30 amino acids deleted (IncD-ΔSS), indicating that the first 30 amino acids contain the secretion signal (SS) required for T3S (Fig. 4A). Samples were probed with an antibody directed against YopN, an endogenous *Yersinia* effector, to confirm that the overexpression of these pro-

teins did not interfere with secretion of endogenous *Yersinia* effectors (Fig. 4B). These data show that IncD can be secreted in a T3SS-dependent manner by *Y. pseudotuberculosis* and suggest that it may also be secreted in a T3SS-dependent manner in *C. trachomatis*.

Secretion of recombinant products from *C. trachomatis*. To examine the secretion of recombinant proteins in *C. trachomatis*, we constructed fusion proteins with a C-terminal Flag tag. *C. trachomatis* was transformed with pBOMB4-Tet containing either an IncD-Flag fusion, IncD-ΔSS-Flag, NrdB-Flag, or CPAF-Flag and examined for localization of the expressed product at 24 h postinfection (Fig. 5A). The IncD-Flag protein was secreted and appropriately localized in the inclusion membrane; however, IncD-ΔSS-Flag lacking the type III secretion signal was retained within the chlamydiae. The negative-control substrate, NrdB-Flag, was also retained by the chlamydiae and not secreted. CPAF-Flag was also largely retained within the chlamydiae but also clearly observed in the cytosol. Immunoblotting with the anti-Flag antibody confirmed that the fusion proteins were being properly expressed (Fig. 5B). These data indicate that fusion proteins can be expressed by chlamydiae and properly localized in infected cells.

Inclusion membrane protein IncD is secreted to the inclusion membrane and is exposed to the cytosol. To confirm type III secretion and exposure of IncD to the cytosol, IncD, IncD-ΔSS, and NrdB were constructed with C-terminal fusions to adenylate cyclase (CyaA) and expressed in *C. trachomatis* L2 (Fig. 6). Appropriate localization of IncD-CyaA in the inclusion membrane was confirmed by immunofluorescence (Fig. 6A). As with the Flag-tagged fusions, IncD lacking the secretion signal and NrdB were

FIG 6 Cytosolic exposure of proteins secreted by *C. trachomatis* determined by CyaA. HeLa cells were infected with *C. trachomatis* L2 transformed with pBOMB4-Tet-IncD-CyaA, pBOMB4-Tet-IncD- Δ SS-CyaA, or pBOMB4-Tet-NrdB-CyaA for 24 h before fixation with 4% paraformaldehyde for immunofluorescence (A) or lysed in 2 $\times$ SDS-PAGE buffer for immunoblotting (B). Samples were probed with anti-IncD or anti-CyaA antibody, followed by a secondary antibody of Alexa 594 (red) and DAPI (blue) (A) or HRP (B). The approximate sizes for CyaA-tagged proteins are as follows: IncD (60.7 kDa), IncD- Δ SS (57.3 kDa), NrdB (84.2 kDa), and CPAF (111.7 kDa). For the cAMP assay, HeLa cells were infected for 24 h in the presence of 500 μ M IBMX and 100 μ M Ro 20-1724, at which time the cAMP assay was performed. Fold induction of cAMP was calculated relative to uninfected cells. *t* test analysis indicates the pBOMB4-Tet-IncD-CyaA cAMP induction is significantly higher than uninfected, IncD- Δ SS and NrdB cAMP levels ($P < 0.001$; $n = 3$). Bar, 10 μ m.

retained within the chlamydiae. Immunoblotting confirmed the expression of each of the constructs (Fig. 6B). cAMP assays demonstrated an increase in cAMP levels in cells infected with IncD-CyaA expressing strains, indicating exposure of CyaA to the cytosol (Fig. 6C). Consistent with the antibody localization studies described above, IncD- Δ SS-CyaA- and NrdB-CyaA-expressing

strains did not demonstrate cAMP levels significantly above background levels.

We also examined the localization of chlamydial proteins tagged with a GSK (glycogen synthase kinase) tag as an alternative means to detect cytosolic exposure. The GSK tag contains 13 amino acids from the glycogen synthase kinase protein and is phosphorylated upon exposure to the cytosol (43). The IncD-GSK fusion protein localizes to the inclusion membrane and is phosphorylated, while the IncD- Δ SS-GSK and NrdB-GSK fusion proteins are retained within the chlamydiae and are not phosphorylated (Fig. 7A). To confirm that the IncD-GSK protein is secreted and exposed to the cytosol, we performed SDS-PAGE and immunoblotted with either anti-GSK or anti-phospho-GSK antibodies (Fig. 7B). The IncD-GSK construct was phosphorylated confirming its exposure to the cytosol.

As additional confirmation of the secretion and exposure of the C-terminal reporters to the cytosol, cells infected with *C. trachomatis* transformed with IncD-Flag or the vector alone were microinjected with anti-Flag monoclonal antibodies at 3 h postinfection. The cultures were then incubated for an additional 21 h and fixed with paraformaldehyde before addition of DyLight 594-conjugated anti-mouse IgG secondary antibody (Fig. 8). Circumferential staining of the inclusion membrane was observed in the IncD-Flag expressing strain but not the negative control, confirming the cytosolic exposure of IncD.

DISCUSSION

Until very recently, chlamydiae had been considered genetically intractable (44, 45). The recently described methods of stably introducing recombinant DNA into *C. trachomatis* (27) offers unprecedented opportunities to apply modern molecular biology technologies to studies of chlamydial biology and pathogenesis. We describe here a shuttle vector designed for the expression of chlamydial secreted effector proteins tagged with a variety of reporters to demonstrate appropriate localization of two secreted chlamydial effector proteins which are translocated by distinct mechanisms. Although chlamydial genomes encode components of both type II and type III secretion machinery (16–19), the lack of genetic tools coupled with their obligate intracellular lifestyle has relegated study of secreted effectors to the use of surrogate systems (34, 40, 41). With the ability to express tagged versions of putative chlamydial effectors, it is now possible to demonstrate secretion from chlamydia and show localization by immunofluorescence or exposure to the cytosol biochemically through adenylate cyclase activity or phosphorylation of GSK by host kinases.

Studies of chlamydial type III secreted effectors in heterologous systems have utilized intact genes (17, 34, 42, 46), or in many cases, fusions of the first 20 to 100 N-terminal amino acids to various reporters (23, 34, 40, 47, 48). Type III secretion signals occur in the N terminus of the protein but lack primary sequence similarity (49). Computational predictions of type III secreted effectors indicate that the signal sequence is located within the N-terminal 30 amino acids and show compositional bias (50–52). Similarly, chlamydial IncS lack primary amino acid sequence similarity even in the putative N-terminal signal sequences and the characteristic bilobed hydrophobic domains (23, 24). We show here that the type III secretory signal of IncD in *C. trachomatis* occurs within the first 30 residues, that it is essential for secretion, and that the hydrophobic domain alone is insufficient for inclusion membrane localization.

FIG 7 GSK assay indicates *C. trachomatis* proteins can be secreted and gain access to the cytosol. HeLa cells were infected with *C. trachomatis* L2 transformed with pBOMB4-Tet-IncD-GSK, pBOMB4-Tet-IncD- Δ SS-GSK, or pBOMB4-Tet-NrdB-GSK for 24 h before fixation with 4% paraformaldehyde for immunofluorescence (A) or lysed in 2 $\times$ SDS-PAGE buffer for immunoblotting (B). (A) Samples were probed with α -GSK or α -Phospho-GSK antibody, followed by a secondary antibody of Alexa 594 (red) and DAPI (blue). The α -phospho-GSK antibody shows some cross-reactivity with the endogenous phospho-GSK in the nucleus. Bar, 10 μ m. (B) Immunoblots were probed with α -GSK tag or α -phospho-GSK antibody followed by an HRP-conjugated secondary antibody. Approximate sizes for the GSK-IncD, GSK-IncD- Δ SS-GSK, and GSK-NrdB are 18.7, 15.3, and 42.2 kDa, respectively.

Chlamydiae have been known for some time now to have the capacity for genetic recombination. Many strains of *C. suis*, a pathogen of swine, contain a transposon-mediated tetracycline resistance cassette integrated into the chromosome (53, 54). These resistance cassettes share a high degree of sequence similarity to resistance plasmids from a variety of bacterial species and may have been selected

FIG 8 Microinjection confirms cytosolic exposure of the IncD-Flag protein. Monolayers of HeLa cells were infected with *C. trachomatis* L2 transformed with pBOMB4R-IncD-Flag, or pBOMB4R for 3 h before the anti-Flag antibody was microinjected into cells. The cultures were incubated for an additional 21 h before fixation with 4% paraformaldehyde for immunofluorescence. Samples were probed with a secondary antibody of Alexa 594 (red), GFP is expressed from the plasmid in transformed chlamydia (green), and Nomarski (differential interference contrast) images are also shown. Bar, 10 μ m.

for in *C. suis* as a result of exposure to antibiotic containing feed (53, 54). Subsequently, recombination between *C. trachomatis* stains in coinfecting cultures was demonstrated (55–58). Although chlamydiae appeared to have the capacity for recombination, achieving this under controlled conditions in the laboratory proved more difficult. Transformation of EBs by electroporation and selection for chloramphenicol resistance was successful but unstable (59). Homologous recombination of the *C. psittaci* rRNA operon with an electroporated synthetic 16S rRNA allele which imparted resistance to kasugamycin (Ksm) and spectinomycin (Spc) was also shown (60). A major advance in chlamydial genetics was provided by Wang et al. (27), who described a robust plasmid-based genetic transformation system that involves transforming EBs with calcium chloride and selection with penicillin (27). Since the development of these techniques, multiple groups have expanded the genetic tools available by modifying vectors to include various fluorescent proteins, alternative selection markers, and inducible promoters (32, 38, 61). Another approach used was the use of dendrimers to deliver DNA and transform chlamydiae (62, 63). The ability to mutate and replace the cryptic chlamydial plasmid has led to the identification of essential and nonessential genes, as well as defining *pgp4* as a regulator of virulence-associated gene expression (31, 64).

Methods to target and knock out specific chlamydial genes using chemical mutagenesis have been described recently (65, 66) and dendrimers have been used to deliver antisense oligonucleotides to specifically knock down target genes (67). As molecular tools to genetically modify chlamydiae are rapidly being developed, additional means to directly target genes through homologous recombination are likely to become available. Thus, it may soon be possible to disrupt type III secretion in chlamydiae, although the obligate intracellular character of the bacteria may be a severe limitation if secreted effectors are essential for chlamydial survival.

Previous limitations on genetic tools for chlamydiae were an incentive for genomic sequencing as a means to identify biovar-specific virulence determinants. As a consequence, there is good

coverage and availability of chlamydial genomes (19, 68–77). In addition, there are now several *in silico* predictive tools for the identification of putative secreted effector proteins (23, 51, 52, 78). The pBOMB4 vector permits expression of chlamydial proteins under the control of either constitutive or inducible promoters and allows the detection of tagged bacterial proteins by immunofluorescence, immunoblotting, or biochemically. We have applied several methods of confirming that bacterial proteins gain access to the cytosol, including CyaA, GSK, and microinjection assays. With the development of powerful new techniques to genetically manipulate chlamydiae, the field has a wealth of new research avenues to pursue.

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REFERENCES

- Burton MJ, Mabey DC. 2009. The global burden of trachoma: a review. *PLoS Negl. Trop. Dis.* 3:e460. <http://dx.doi.org/10.1371/journal.pntd.0000460>.
- Bébéar C, de Barbeyrac B. 2009. Genital *Chlamydia trachomatis* infections. *Clin. Microbiol. Infect.* 15:4–10. <http://dx.doi.org/10.1111/j.1469-0691.2008.02647.x>.
- Schachter J. 1999. Infection and disease epidemiology, p 139–169. In Stephens RS (ed), *Chlamydia: intracellular biology, pathogenesis, and immunity*. ASM Press, Washington, DC.
- Grayston JT. 1992. Infections caused by *Chlamydia pneumoniae* strain TWAR. *Clin. Infect. Dis.* 15:757–761. <http://dx.doi.org/10.1093/clind/15.5.757>.
- Beeckman DS, Vanrompay DC. 2009. Zoonotic *Chlamydophila psittaci* infections from a clinical perspective. *Clin. Microbiol. Infect.* 15:11–17. <http://dx.doi.org/10.1111/j.1469-0691.2008.02669.x>, <http://dx.doi.org/10.1111/j.1469-0691.2009.03054.x>.
- Wheelhouse N, Longbottom D. 2012. Endemic and emerging chlamydial infections of animals and their zoonotic implications. *Transbound. Emerg. Dis.* 59:283–291. <http://dx.doi.org/10.1111/j.1865-1682.2011.01274.x>.
- Moulder JW. 1991. Interaction of chlamydiae and host cells in vitro. *Microbiol. Rev.* 55:143–190.
- Abdelrahman YM, Belland RJ. 2005. The chlamydial developmental cycle. *FEMS Microbiol. Rev.* 29:949–959. <http://dx.doi.org/10.1016/j.femsre.2005.03.002>.
- Scidmore MA, Fischer ER, Hackstadt T. 2003. Restricted fusion of *Chlamydia trachomatis* vesicles with endocytic compartments during the initial stages of infection. *Infect. Immun.* 71:973–984. <http://dx.doi.org/10.1128/IAI.71.2.973-984.2003>.
- Scidmore MA, Rockey DD, Fischer ER, Heinzen RA, Hackstadt T. 1996. Vesicular interactions of the *Chlamydia trachomatis* inclusion are determined by chlamydial early protein synthesis rather than route of entry. *Infect. Immun.* 64:5366–5372.
- Betts HJ, Wolf K, Fields KA. 2009. Effector protein modulation of host cells: examples in the *Chlamydia* spp. arsenal. *Curr. Opin. Microbiol.* 12:81–87. <http://dx.doi.org/10.1016/j.mib.2008.11.009>.
- Fields KA, Hackstadt T. 2002. The chlamydial inclusion: escape from the endocytic pathway. *Annu. Rev. Cell Dev. Biol.* 18:221–245. <http://dx.doi.org/10.1146/annurev.cellbio.18.012502.105845>.
- Hackstadt T, Fischer ER, Scidmore MA, Rockey DD, Heinzen RA. 1997. Origins and functions of the chlamydial inclusion. *Trends Microbiol.* 5:288–293. [http://dx.doi.org/10.1016/S0966-842X\(97\)01061-5](http://dx.doi.org/10.1016/S0966-842X(97)01061-5).
- Cocchiari JL, Valdivia RH. 2009. New insights into *Chlamydia* intracellular survival mechanisms. *Cell Microbiol.* 11:1571–1578. <http://dx.doi.org/10.1111/j.1462-5822.2009.01364.x>.
- Zhong G. 2009. Killing me softly: chlamydial use of proteolysis for evading host defenses. *Trends Microbiol.* 17:467–474. <http://dx.doi.org/10.1016/j.tim.2009.07.007>.
- Chen D, Lei L, Lu C, Flores R, DeLisa MP, Roberts TC, Romesberg FE, Zhong G. 2010. Secretion of the chlamydial virulence factor CPAF requires the Sec-dependent pathway. *Microbiology* 156:3031–3040. <http://dx.doi.org/10.1099/mic.0.040527-0>.
- Fields KA, Mead D, Dooley CA, Hackstadt T. 2003. *Chlamydia trachomatis* type III secretion: evidence for a functional apparatus during early-cycle development. *Mol. Microbiol.* 48:671–683. <http://dx.doi.org/10.1046/j.1365-2958.2003.03462.x>.
- Hsia RC, Pannekoek Y, Ingerowski E, Bavoil PM. 1997. Type III secretion genes identify a putative virulence locus of *Chlamydia*. *Mol. Microbiol.* 25:351–359. <http://dx.doi.org/10.1046/j.1365-2958.1997.4701834.x>.
- Stephens RS, Kalman S, Lammel C, Fan J, Marathe R, Aravind L, Mitchell W, Olinger L, Tatusov RL, Zhao Q, Koonin EV, Davis RW. 1998. Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. *Science* 282:754–759. <http://dx.doi.org/10.1126/science.282.5389.754>.
- Hueck CJ. 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol. Mol. Biol. Rev.* 62:379–433.
- Hackstadt T, Scidmore-Carlson MA, Shaw EI, Fischer ER. 1999. The *Chlamydia trachomatis* IncA protein is required for homotypic vesicle fusion. *Cell Microbiol.* 1:119–130. <http://dx.doi.org/10.1046/j.1462-5822.1999.00012.x>.
- Bannantine JP, Griffiths RS, Viratyosin W, Brown WJ, Rockey DD. 2000. A secondary structure motif predictive of protein localization to the chlamydial inclusion membrane. *Cell Microbiol.* 2:35–47. <http://dx.doi.org/10.1046/j.1462-5822.2000.00029.x>.
- Dehoux P, Flores R, Dauga C, Zhong G, Subtil A. 2011. Multi-genome identification and characterization of chlamydiae-specific type III secretion substrates: the Inc proteins. *BMC Genomics* 12:109. <http://dx.doi.org/10.1186/1471-2164-12-109>.
- Lutter EI, Martens C, Hackstadt T. 2012. Evolution and conservation of predicted inclusion membrane proteins in chlamydiae. *Comp. Funct. Genomics* 2012:362104. <http://dx.doi.org/10.1016/j.celrep.2013.04.027>.
- Li Z, Chen C, Chen D, Wu Y, Zhong Y, Zhong G. 2008. Characterization of fifty putative inclusion membrane proteins encoded in the *Chlamydia trachomatis* genome. *Infect. Immun.* 76:2746–2757. <http://dx.doi.org/10.1128/IAI.00010-08>.
- Toh H, Miura K, Shirai M, Hattori M. 2003. In silico inference of inclusion membrane protein family in obligate intracellular parasites chlamydiae. *DNA Res.* 10:9–17. <http://dx.doi.org/10.1093/dnares/10.1.9>.
- Wang Y, Kahane S, Cutcliffe LT, Skilton RJ, Lambden PR, Clarke IN. 2011. Development of a transformation system for *Chlamydia trachomatis*: restoration of glycogen biosynthesis by acquisition of a plasmid shuttle vector. *PLoS Pathog.* 7:e1002258. <http://dx.doi.org/10.1371/journal.ppat.1002258>.
- Caldwell HD, Kromhout J, Schachter J. 1981. Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis*. *Infect. Immun.* 31:1161–1176.
- Furness G, Graham DM, Reeve P. 1960. The titration of trachoma and inclusion blennorrhoea viruses in cell cultures. *J. Gen. Microbiol.* 23:613–619. <http://dx.doi.org/10.1099/00221287-23-3-613>.
- Voth DE, Beare PA, Howe D, Sharma UM, Samoilis G, Cockrell DC, Omsland A, Heinzen RA. 2011. The *Coxiella burnetii* cryptic plasmid is enriched in genes encoding type IV secretion system substrates. *J. Bacteriol.* 193:1493–1503. <http://dx.doi.org/10.1128/JB.01359-10>.
- Song L, Carlson JH, Whitmire WM, Kari L, Virtaneva K, Sturdevant DE, Watkins H, Zhou B, Sturdevant GL, Porcella SF, McClarty G, Caldwell HD. 2013. *Chlamydia trachomatis* plasmid-encoded Pgp4 is a transcriptional regulator of virulence-associated genes. *Infect. Immun.* 81:636–644. <http://dx.doi.org/10.1128/IAI.01305-12>.
- Agaisse H, Derre I. 2013. A *C. trachomatis* cloning vector and the generation of *C. trachomatis* strains expressing fluorescent proteins under the control of a *C. trachomatis* promoter. *PLoS One* 8:e57090. <http://dx.doi.org/10.1371/journal.pone.0057090>.
- Heinzen RA, Hackstadt T. 1997. The *Chlamydia trachomatis* parasitophorous vacuolar membrane is not passively permeable to low-molecular-weight compounds. *Infect. Immun.* 65:1088–1094.
- Fields KA, Hackstadt T. 2000. Evidence for the secretion of *Chlamydia trachomatis* CopN by a type III secretion mechanism. *Mol. Microbiol.* 38:1048–1060. <http://dx.doi.org/10.1046/j.1365-2958.2000.02212.x>.
- Day JB, Plano GV. 1998. A complex composed of SycN and YscB func-

- tions as a specific chaperone for YopN in *Yersinia pestis*. *Mol. Microbiol.* 30:777–788. <http://dx.doi.org/10.1046/j.1365-2958.1998.01110.x>.
36. Belland RJ, Zhong G, Crane DD, Hogan D, Sturdevant D, Sharma J, Beatty WL, Caldwell HD. 2003. Genomic transcriptional profiling of the developmental cycle of *Chlamydia trachomatis*. *Proc. Natl. Acad. Sci. U. S. A.* 100:8478–8483. <http://dx.doi.org/10.1073/pnas.1331135100>.
 37. Shaw EI, Dooley CA, Fischer ER, Scidmore MA, Fields KA, Hackstadt T. 2000. Three temporal classes of gene expression during the *Chlamydia trachomatis* developmental cycle. *Mol. Microbiol.* 37:913–925. <http://dx.doi.org/10.1046/j.1365-2958.2000.02057.x>.
 38. Wickstrum J, Sammons LR, Restivo KN, Hefty PS. 2013. Conditional gene expression in *Chlamydia trachomatis* using the Tet system. *PLoS One* 8:e76743. <http://dx.doi.org/10.1371/journal.pone.0076743>.
 39. Bianchi A, Scieux C, Salmeron CM, Casin I, Perol Y. 1988. Rapid determination of MICs of 15 antichlamydial agents by using an enzyme immunoassay (*Chlamydiazyme*). *Antimicrob. Agents Chemother.* 32:1350–1353.
 40. Subtil A, Parsot C, Dautry-Varsat A. 2001. Secretion of predicted Inc proteins of *Chlamydia pneumoniae* by a heterologous type III machinery. *Mol. Microbiol.* 39:792–800. <http://dx.doi.org/10.1046/j.1365-2958.2001.02272.x>.
 41. Ho TD, Starnbach MN. 2005. The *Salmonella enterica* serovar Typhimurium-encoded type III secretion systems can translocate *Chlamydia trachomatis* proteins into the cytosol of host cells. *Infect. Immun.* 73:905–911. <http://dx.doi.org/10.1128/IAI.73.2.905-911.2005>.
 42. Clifton DR, Dooley CA, Grieshaber SS, Carabeo RA, Fields KA, Hackstadt T. 2005. Tyrosine phosphorylation of chlamydial Tarp is species specific and not required for the recruitment of actin. *Infect. Immun.* 73:3860–3868. <http://dx.doi.org/10.1128/IAI.73.7.3860-3868.2005>.
 43. Garcia JT, Ferracci F, Jackson MW, Joseph SS, Pattis I, Plano LR, Fischer W, Plano GV. 2006. Measurement of effector protein injection by type III and type IV secretion systems by using a 13-residue phosphorylatable glycogen synthase kinase tag. *Infect. Immun.* 74:5645–5657. <http://dx.doi.org/10.1128/IAI.00690-06>.
 44. Heuer D, Kneip C, Maurer AP, Meyer TF. 2007. Tackling the intractable: approaching the genetics of *Chlamydiales*. *Int. J. Med. Microbiol.* 297: 569–576. <http://dx.doi.org/10.1016/j.ijmm.2007.03.011>.
 45. Thomson NR, Clarke IN. 2010. *Chlamydia trachomatis*: small genome, big challenges. *Future Microbiol.* 5:555–561. <http://dx.doi.org/10.2217/fmb.10.31>.
 46. Clifton DR, Fields KA, Grieshaber S, Dooley CA, Fischer E, Mead D, Carabeo RA, Hackstadt T. 2004. A chlamydial type III translocated protein is tyrosine phosphorylated at the site of entry and associated with recruitment of actin. *Proc. Natl. Acad. Sci. U. S. A.* 101:10166–10171. <http://dx.doi.org/10.1073/pnas.0402829101>.
 47. Muschiol S, Boncompain G, Vromman F, Dehoux P, Normark S, Henriques-Normark B, Subtil A. 2011. Identification of a family of effectors secreted by the type III secretion system that are conserved in pathogenic *Chlamydiae*. *Infect. Immun.* 79:571–580. <http://dx.doi.org/10.1128/IAI.00825-10>.
 48. Subtil A, Delevoeye C, Balana ME, Tastevin L, Perrinet S, Dautry-Varsat A. 2005. A directed screen for chlamydial proteins secreted by a type III mechanism identifies a translocated protein and numerous other new candidates. *Mol. Microbiol.* 56:1636–1647. <http://dx.doi.org/10.1111/j.1365-2958.2005.04647.x>.
 49. Michiels T, Vanoorteghem JC, Lambert de Rouvroit C, China B, Gustin A, Boudry P, Cornelis GR. 1991. Analysis of *virC*, an operon involved in the secretion of Yop proteins by *Yersinia enterocolitica*. *J. Bacteriol.* 173: 4994–5009.
 50. Arnold R, Brandmaier S, Kleine F, Tischler P, Heinz E, Behrens S, Niinikoski A, Mewes HW, Horn M, Rattei T. 2009. Sequence-based prediction of type III secreted proteins. *PLoS Pathog.* 5:e1000376. <http://dx.doi.org/10.1371/journal.ppat.1000376>.
 51. McDermott JE, Corrigan A, Peterson E, Oehmen C, Niemann G, Cambromne ED, Sharp D, Adkins JN, Samudrala R, Heffron F. 2011. Computational prediction of type III and IV secreted effectors in gram-negative bacteria. *Infect. Immun.* 79:23–32. <http://dx.doi.org/10.1128/IAI.00537-10>.
 52. Samudrala R, Heffron F, McDermott JE. 2009. Accurate prediction of secreted substrates and identification of a conserved putative secretion signal for type III secretion systems. *PLoS Pathog.* 5:e1000375. <http://dx.doi.org/10.1371/journal.ppat.1000375>.
 53. Dugan J, Rockey DD, Jones L, Andersen AA. 2004. Tetracycline resistance in *Chlamydia suis* mediated by genomic islands inserted into the chlamydial inv-like gene. *Antimicrob. Agents Chemother.* 48:3989–3995. <http://dx.doi.org/10.1128/AAC.48.10.3989-3995.2004>.
 54. Dugan J, Andersen AA, Rockey DD. 2007. Functional characterization of IS*cs605*, an insertion element carried by tetracycline-resistant *Chlamydia suis*. *Microbiology* 153:71–79. <http://dx.doi.org/10.1099/mic.0.29253-0>.
 55. DeMars R, Weinfurter J. 2008. Interstrain gene transfer in *Chlamydia trachomatis* in vitro: mechanism and significance. *J. Bacteriol.* 190:1605–1614. <http://dx.doi.org/10.1128/JB.01592-07>.
 56. Demars R, Weinfurter J, Guex E, Lin J, Potucek Y. 2007. Lateral gene transfer in vitro in the intracellular pathogen *Chlamydia trachomatis*. *J. Bacteriol.* 189:991–1003. <http://dx.doi.org/10.1128/JB.00845-06>.
 57. Suchland RJ, Sandoz KM, Jeffrey BM, Stamm WE, Rockey DD. 2009. Horizontal transfer of tetracycline resistance among *Chlamydia* spp. in vitro. *Antimicrob. Agents Chemother.* 53:4604–4611. <http://dx.doi.org/10.1128/AAC.00477-09>.
 58. Jeffrey BM, Suchland RJ, Eriksen SG, Sandoz KM, Rockey DD. 2013. Genomic and phenotypic characterization of in vitro-generated *Chlamydia trachomatis* recombinants. *BMC Microbiol.* 13:142. <http://dx.doi.org/10.1186/1471-2180-13-142>.
 59. Tam JE, Davis CH, Wyrick PB. 1994. Expression of recombinant DNA introduced into *Chlamydia trachomatis* by electroporation. *Can. J. Microbiol.* 40:583–591. <http://dx.doi.org/10.1139/m94-093>.
 60. Binet R, Maurelli AT. 2009. Transformation and isolation of allelic exchange mutants of *Chlamydia psittaci* using recombinant DNA introduced by electroporation. *Proc. Natl. Acad. Sci. U. S. A.* 106:292–297. <http://dx.doi.org/10.1073/pnas.0806768106>.
 61. Xu S, Battaglia L, Bao X, Fan H. 2013. Chloramphenicol acetyltransferase as a selection marker for chlamydial transformation. *BMC Res. Notes* 6:377. <http://dx.doi.org/10.1186/1756-0500-6-377>.
 62. Gerard HC, Mishra MK, Mao G, Wang S, Hali M, Whittum-Hudson JA, Kannan RM, Hudson AP. 2013. Dendrimer-enabled DNA delivery and transformation of *Chlamydia pneumoniae*. *Nanomedicine* 9:996–1008. <http://dx.doi.org/10.1016/j.nano.2013.04.004>.
 63. Kannan RM, Gerard HC, Mishra MK, Mao G, Wang S, Hali M, Whittum-Hudson JA, Hudson AP. 2013. Dendrimer-enabled transformation of *Chlamydia trachomatis*. *Microb. Pathog.* 65C:29–35. <http://dx.doi.org/10.1016/j.micpath.2013.08.003>.
 64. Gong S, Yang Z, Lei L, Shen L, Zhong G. 2013. Characterization of *Chlamydia trachomatis* plasmid-encoded open reading frames. *J. Bacteriol.* 195:3819–3826. <http://dx.doi.org/10.1128/JB.00511-13>.
 65. Kari L, Goheen MM, Randall LB, Taylor LD, Carlson JH, Whitmire WM, Virok D, Rajaram K, Endresz V, McClarty G, Nelson DE, Caldwell HD. 2011. Generation of targeted *Chlamydia trachomatis* null mutants. *Proc. Natl. Acad. Sci. U. S. A.* 108:7189–7193. <http://dx.doi.org/10.1073/pnas.1102229108>.
 66. Nguyen BD, Valdivia RH. 2012. Virulence determinants in the obligate intracellular pathogen *Chlamydia trachomatis* revealed by forward genetic approaches. *Proc. Natl. Acad. Sci. U. S. A.* 109:1263–1268. <http://dx.doi.org/10.1073/pnas.1117884109>.
 67. Mishra MK, Gerard HC, Whittum-Hudson JA, Hudson AP, Kannan RM. 2012. Dendrimer-enabled modulation of gene expression in *Chlamydia trachomatis*. *Mol. Pharm.* 9:413–421. <http://dx.doi.org/10.1021/mp200512f>.
 68. Azuma Y, Hirakawa H, Yamashita A, Cai Y, Rahman MA, Suzuki H, Mitaku S, Toh H, Goto S, Murakami T, Sugi K, Hayashi H, Fukushima H, Hattori M, Kuhara S, Shirai M. 2006. Genome sequence of the cat pathogen, *Chlamydomonas felis*. *DNA Res.* 13:15–23. <http://dx.doi.org/10.1093/dnares/dsi027>.
 69. Carlson JH, Porcella SF, McClarty G, Caldwell HD. 2005. Comparative genomic analysis of *Chlamydia trachomatis* oculotropic and genitotropic strains. *Infect. Immun.* 73:6407–6418. <http://dx.doi.org/10.1128/IAI.73.10.6407-6418.2005>.
 70. Kalman S, Mitchell W, Marathe R, Lammel C, Fan J, Hyman RW, Olinger L, Grimwood J, Davis RW, Stephens RS. 1999. Comparative genomes of *Chlamydia pneumoniae* and *C. trachomatis*. *Nat. Genet.* 21: 385–389. <http://dx.doi.org/10.1038/7716>.
 71. Mojica S, Huot Creasy H, Daugherty S, Read TD, Kim T, Kaltenboeck B, Bavoil P, Myers GS. 2011. Genome sequence of the obligate intracellular animal pathogen *Chlamydia pecorum* E58. *J. Bacteriol.* 193:3690. <http://dx.doi.org/10.1128/JB.00454-11>.
 72. Read TD, Brunham RC, Shen C, Gill SR, Heidelberg JF, White O, Hickey EK, Peterson J, Utterback T, Berry K, Bass S, Linher K, Weidman J, Khouri H, Craven B, Bowman C, Dodson R, Gwinn M,

- Nelson W, DeBoy R, Kolonay J, McClarty G, Salzberg SL, Eisen J, Fraser CM. 2000. Genome sequences of *Chlamydia trachomatis* MoPn and *Chlamydia pneumoniae* AR39. *Nucleic Acids Res.* 28:1397–1406. <http://dx.doi.org/10.1093/nar/28.6.1397>.
73. Read TD, Muers GS, Brunham RC, Nelson WC, Paulsen IT, Heidelberg JF, Holtzapple E, Khouri H, Federova NB, Carty HA, Umayam LA, Haft DH, Peterson J, Beanan MJ, White O, Salzberg SL, Hsia R, McClarty G, Rank RG, Bavoil PM, Fraser CM. 2003. Genome sequence of *Chlamydophila caviae* (*Chlamydia psittaci* GPIC): examining the role of niche-specific genes in the evolution of the *Chlamydiaceae*. *Nucleic Acids Res.* 31:2134–2147. <http://dx.doi.org/10.1093/nar/gkg321>.
 74. Thomson NR, Holden MT, Carder C, Lennard N, Lockey SJ, Marsh P, Skipp P, O'Conner CD, Goodhead I, Norbertzake H, Harris B, Ormond D, Rance R, Quail MA, Parkhill J, Stephens RS, Clarke IN. 2008. *Chlamydia trachomatis*: genome sequence analysis of lymphogranuloma venereum isolates. *Genome Res.* 18:161–171.
 75. Thomson NR, Yeats C, Bell K, Holden MT, Bentley SD, Livingstone M, Cerdeno-Tarraga AM, Harris B, Doggett J, Ormond D, Mungall K, Clarke K, Feltwell T, Hance Z, Sanders M, Quail MA, Price C, Barrell BG, Parkhill J, Longbottom D. 2005. The *Chlamydophila abortus* genome sequence reveals an array of variable proteins that contribute to interspecies variation. *Genome Res.* 15:629–640. <http://dx.doi.org/10.1101/gr.3684805>.
 76. Unemo M, Clarke IN. 2011. The Swedish new variant of *Chlamydia trachomatis*. *Curr. Opin. Infect. Dis.* 24:62–69. <http://dx.doi.org/10.1097/QCO.0b013e32834204d5>.
 77. Voigt A, Schofl G, Saluz HP. 2012. The *Chlamydia psittaci* genome: a comparative analysis of intracellular pathogens. *PLoS One* 7:e35097. <http://dx.doi.org/10.1371/journal.pone.0035097>.
 78. Hovis KM, Mojica S, McDermott JE, Pedersen L, Simhi C, Rank RG, Myers GS, Ravel J, Hsia RC, Bavoil PM. 2013. Genus-optimized strategy for the identification of chlamydial type III secretion substrates. *Pathog. Dis.* 69:213–222. <http://dx.doi.org/10.1111/2049-632X.12070>.