

The *Salmonella enterica* Serovar Typhimurium-Encoded Type III Secretion Systems Can Translocate *Chlamydia trachomatis* Proteins into the Cytosol of Host Cells

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Chlamydia trachomatis is an obligate, intracellular pathogen that is a major cause of preventable blindness and infertility worldwide. Although the published genome sequence suggests that *C. trachomatis* encodes a type III secretion system, the lack of genetic tools for studying *Chlamydia* has hindered the examination of this potentially important class of virulence genes. We have developed a technique to identify *Chlamydia* proteins that can be translocated into the host cell cytoplasm by a type III secretion system. We have selected several *Chlamydia* proteins and tagged them with a multiple peptide motif element called F8M4. Epitopes contained in the F8M4 tag allow us to use tools corresponding to different arms of the adaptive immune system to detect the expression and translocation of these proteins by *Salmonella enterica* serovar Typhimurium. In particular, CD8⁺-T-cell reactivity can be used to detect the translocation of F8M4-tagged proteins into the cytoplasm of host cells. We have found that CD8⁺-T-cell activity assays are sensitive enough to detect translocation of even a small amount of F8M4-tagged protein. We have used CD8⁺-T-cell activity to show that CopN, a *Chlamydia* protein previously shown to be translocated by *Yersinia* type III secretion, can be translocated by the *Salmonella* pathogenicity island 1 (SPI-1) type III secretion system. Additionally, we demonstrate that CopD and Pkn5, two *Chlamydia* proteins hypothesized to be substrates of a type III secretion system, are translocated via the SPI-2 type III secretion system of serovar Typhimurium. The epitope tag system described here can be used more generally to examine the expression and subcellular compartmentalization of bacterial proteins deployed during the interaction of pathogens with mammalian cells.

Chlamydia trachomatis causes a wide range of diseases throughout the world. Ocular infection with *C. trachomatis* results in millions of cases of severe visual impairment and is the leading cause of preventable blindness in developing nations. In developed countries, *C. trachomatis* is one of the most common bacterial causes of sexually transmitted disease. If left untreated, these genital infections can result in infertility. In the United States alone, *C. trachomatis* causes approximately 3 million cases of sexually transmitted disease per year (7).

The chlamydiae are gram-negative, obligate, intracellular pathogens that reside within host cells in a membrane-bound vacuole called the inclusion. *Chlamydia* undergoes a regulated developmental cycle (18). The elementary body is the infectious but metabolically inactive form of *Chlamydia*. The elementary body invades the host cell, where it differentiates into a reticulate body. The reticulate body is the noninfectious, metabolically active, replicative form. After undergoing between 100 and 1,000 rounds of replication, the reticulate bodies differentiate back into elementary bodies prior to lysis of the host cell.

Several gram-negative pathogens encode type III secretion systems which translocate a number of bacterial proteins, or effectors, directly into the host cell cytoplasm. The published genome sequences of several *Chlamydia* species suggest that these bacteria encode proteins homologous to components of

type III secretion systems from other bacteria (3, 4, 16, 18). Unlike the tightly clustered genes encoding type III secretion systems in other organisms, the type III secretion genes of *Chlamydia* are located in at least three different locations or subclusters on the chromosome (12, 19). Several *Chlamydia* proteins are hypothesized to be substrates for type III secretion. The CopB (CT578) and CopD (CT579) proteins are encoded within one subcluster and have been hypothesized to be homologous to the *Yersinia* YopB and YopD proteins, respectively, based on predicted secondary structure, hydrophobicity, and gene operon organization (19). Another type III secretion subcluster encodes a putative serine/threonine kinase, Pkn5, which may also be translocated by a type III secretion system (19).

Since there are currently no means of genetically manipulating *Chlamydia* spp., studies of the *Chlamydia* proteins which are thought to be translocated have been conducted by using the type III secretion systems of other organisms (3, 20). Several *Chlamydia* proteins are secreted by the type III secretion systems of *Shigella flexneri* and *Yersinia enterocolitica*. The *C. trachomatis* proteins CopN, a homologue of the *Yersinia* effector YopN, and IncC, a protein which localizes to the inclusion membrane, are secreted by a *Yersinia* type III secretion system. This fact was demonstrated by tagging the CopN and IncC proteins with an antibody epitope. By using immunofluorescence microscopy and immunoblotting, these studies showed that these proteins were secreted into culture supernatants by a *Yersinia* type III secretion system (3, 4). The *Chlamydia pneumoniae* proteins IncA, IncB, and IncC and a number of other inclusion membrane proteins are secreted by the type III

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TABLE 1. Bacterial strains

Strain	Genotype	Source or reference
<i>E. coli</i>		
DH5 α pir ⁺	<i>endA1 hsdR17 glnV44 thi-1 recA gyrA Δ(lacIZYA-argF)U169 deoR [ϕ80dlacΔ(lacZ)M15]</i>	
EC100pir ⁺	<i>mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK rpsL nupG pir⁺ (DHFR)</i>	Epicenter
TOP10F'	<i>F' lacI^q Tn10/recA1 endA1 mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80dlacZΔM15 ΔlacX74 araD139 Δ(ara, leu)7697 galU galK rpsL nupG</i>	Invitrogen
Serovar Typhimurium 14028	Wild-type serovar Typhimurium	American Type Culture Collection
TDH111	<i>F' (lacI^q Tn10)/14028</i>	This study
AWM430	<i>invA::cat</i>	21
TDH123	<i>invA::cat/14028</i>	This study
MJW129	<i>ssrB::cat</i>	23
TDH124	<i>ssrB::cat/14028</i>	This study

secretion system of *Shigella flexneri* (20). In that study, Inc proteins were fused to an adenylate cyclase reporter. These protein fusions were translocated by the *Shigella* type III secretion system into the host cell cytoplasm, where the reporter protein fusion exhibited enzymatic activity. These studies demonstrated that *Chlamydia* effectors highly expressed in heterologous enteric gram-negative bacteria organisms could be translocated into the cytoplasm of eukaryotic cells by type III secretion systems.

Here, we have used the type III secretion systems of *Salmonella enterica* serovar Typhimurium to identify additional *C. trachomatis* proteins which can be translocated into the host cell cytoplasm. Like *C. trachomatis*, serovar Typhimurium survives and replicates within host cell vacuoles. To detect the translocation of these proteins, we have tagged several proteins with a known CD8⁺-T-cell epitope. When these tagged proteins are delivered into the host cell cytoplasm, they are processed and presented in the context of major histocompatibility class I (MHC-I) molecules to CD8⁺ T cells. We have used the activity of CD8⁺ T cells specific for the epitope tag to detect the delivery of these epitope-tagged bacterial proteins into the host cell cytoplasm. Using CD8⁺ T cells as a probe, we demonstrated that the *C. trachomatis* proteins CopN, CopD, and Pkn5 were translocated by the type III secretion systems of serovar Typhimurium.

MATERIALS AND METHODS

Bacterial strains, media, and genetic techniques. The bacterial strains used in this study are described in Table 1. All serovar Typhimurium strains are isogenic derivatives of serovar Typhimurium 14028s (American Type Culture Collection). Bacterial strains were grown using Luria-Bertani (LB) medium. Ampicillin (100 μ g/ml), tetracycline (25 μ g/ml), kanamycin (50 μ g/ml), and chloramphenicol (20 μ g/ml) were used as selective antibiotics. To control the expression of the F8M4-tagged proteins, we generated TDH111, a serovar Typhimurium strain which expresses the constitutive *lac* repressor in single copy. The expression of F8M4-tagged proteins under the transcriptional control of the *lac* promoter was induced by growing the bacteria in medium containing 10 mM isopropyl- β -D-thiogalactopyranoside (IPTG; Calbiochem). We used previously described P22 transduction methods to transfer genetic markers between serovar Typhimurium

A. F8M4



B. TnF8M4

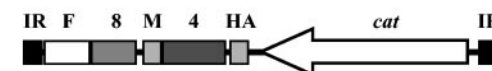


FIG. 1. F8M4 and Tn5-F8M4 constructs. (A) The F8M4 tag was constructed to incorporate the FlAsH motif (F), the OVA_{257–264} CD8⁺-T-cell epitope (8), the *c-myc* epitope (M), and the OVA_{323–339} CD4⁺-T-cell epitope (4) flanked by the inverted repeats (IR) recognized by Tn5 transposase. (B) The TnF8M4 construct was designed to also include the HA epitope and the chloramphenicol acetyltransferase gene (*cat*) between the OVA_{323–339} CD4⁺-T-cell epitope and a Tn5 inverted repeat.

strains (24). Plasmids were transformed into bacterial strains by using standard methods of chemical competence (10) or an electroporation apparatus (Bio-Rad).

DNA sequence and DNA oligomers. DNA sequences were analyzed by using Wisconsin Package (Genetics Computer Group, Inc.) and pDRAW32 (Academy Software) software programs. PCRs were performed by using *Taq* polymerase (Invitrogen) or PfuTurbo polymerase (Stratagene) according to the manufacturer's instructions with serovar Typhimurium 14028 chromosomal DNA or the boiled *C. trachomatis* serovar D or L2 organism as template. Oligonucleotide primers were synthesized by Integrated DNA Technologies.

Construction of the F8M4 epitope tag. The multiple epitope tag was created with the oligomers F8M4 P1F, F8M4 P2Rev, F8M4 P3F, and F8M4 P4Rev. Each oligomer was resuspended in STE buffer (10 mM Tris [pH 8.0], 50 mM NaCl, 1 mM EDTA). Pairs of oligomers (F8M4 P1F and F8M4 P2Rev; F8M4 P3F and F8M4 P4Rev) were annealed according to the manufacturer's instructions. Each annealed oligomer pair was digested by using EcoRI (New England BioLabs). The two oligomer pairs were then ligated by using T4 DNA ligase (New England BioLabs) to generate the F8M4 element (Fig. 1A).

We generated two different F8M4 plasmids for tagging proteins of interest. We used pTDH302 to tag proteins by directly cloning them into the plasmid. pTDH302 carries the F8M4 element just downstream of a multiple cloning site, adding a C-terminal tag to the protein of interest as outlined in Fig. 2A. The second plasmid, pTDH313, contains an additional sequence immediately downstream of the CD4⁺-T-cell epitope of F8M4 encoding the hemagglutinin (HA) epitope and chloramphenicol acetyltransferase (*cat*) (Fig. 1B). This generated the chloramphenicol-resistant transposon TnF8M4. The Tn5-F8M4 transposon used in the EZ::TN in vitro transposition assay was amplified by PCR using the pTDH313 plasmid as template. With these constructs, we generated F8M4-tagged protein fusions either by directly cloning genes on the pTDH302 plasmid or by transposing the Tn5-F8M4 PCR product amplified from pTDH313 onto a plasmid containing the target *C. trachomatis* gene.

Plasmids. The majority of the plasmids used in this study were generated by cloning DNA amplified by PCR onto the plasmid vector. All restriction enzymes and DNA modification enzymes (T4 polynucleotide kinase and calf intestinal alkaline phosphatase) were obtained from New England BioLabs. The pTDH302 plasmid was generated by annealing the F8M4 P1, P2 Rev, P3F, and P4 Rev oligomers and cloning the double-stranded DNA into the pCR2.1-TOPO vector by using a TOPO TA cloning kit (Invitrogen). The resulting plasmid encodes a LacZ_{1–26} protein fusion to the F8M4 tag (Fig. 2A). pTDH14 was constructed by amplifying the 189 bp upstream of and the first 684 bp of the *slrP* gene by PCR with the primers slrP P1 and slrP P2R and cloning the gene in frame with the F8M4 epitope tag of pTDH313 (Fig. 2B). The entire open reading frame of the *C. trachomatis* *copN* gene was amplified by PCR with the primers 089 P1 and 089 P3R and cloned upstream of the F8M4 epitope tag to generate pTDH04 (Fig. 2C). pTDH203 was obtained by cloning the entire *copB* gene and the first 660 bp of *copD* into the pTDH302 plasmid. The *copB* and *copD* genes were amplified by PCR with the primers 578 P1 and 579 P1R (Fig. 2D). The pTDH206 plasmid includes the first 669 bp of *pkn5* which was amplified by PCR with the primers

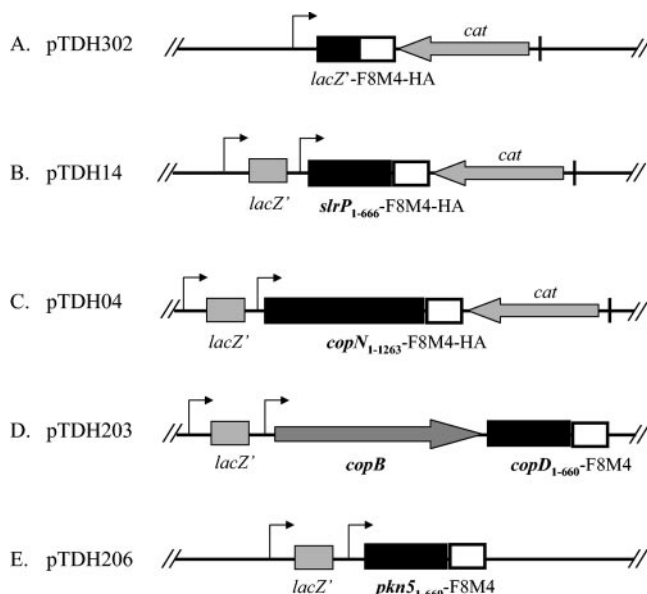


FIG. 2. F8M4-tagged protein fusion constructs.

672 P1 and 673 P1R (Fig. 2E). The resulting fragment was cloned into the pTDH302 vector.

EZ::TN in vitro transposition of TnF8M4. Transposition of TnF8M4 can also result in F8M4-tagged *C. trachomatis* protein fusions. The *Chlamydia copN* gene was amplified by PCR with the primers 089 P1 and 089 P3R. The resulting fragment was cloned into the pCR2.1-TOPO vector by using a TOPO TA cloning kit (Invitrogen), resulting in pTDH01. This plasmid was used as the target for Tn5-F8M4 (EPICENTRE) transposition according to the manufacturer's instructions. We incubated Tn5-F8M4, pTDH01, and Tn5 transposase and transformed this transposition reaction into electrocompetent EC100pir⁺ cells. Thirty-seven chloramphenicol-resistant transformants that presumably contained insertions on the *copN* plasmid pTDH01 were obtained. We isolated plasmids from 13 of these colonies and sequenced the plasmid DNA with a primer which anneals to sequence within the transposable element. Sequence analysis showed that the Tn5-F8M4 insertions were distributed randomly along the length of the pTDH01 plasmid in both orientations.

Immunoblot analysis. To generate whole cell lysates for immunoblot analysis, bacteria were concentrated 10-fold in sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer and boiled for 3 to 5 min. After the samples were centrifuged for 1 min at 13,000 × *g*, 12 µl of supernatant was loaded on 12.5% polyacrylamide gels. After electrophoresis, the protein gels were blotted onto nitrocellulose (Trans-Blot transfer medium; Bio-Rad) for 1 h by using a semidry transblot apparatus (Bio-Rad). Proteins were visualized after blotting by staining the nitrocellulose with 0.1% Ponceau S (Sigma) and 5% acetic acid. After the blots were destained in distilled water, they were blocked with 5% dry milk in phosphate-buffered saline (PBS) for 1 h. Mouse anti-*c-myc* antibody (1:5,000; Invitrogen) was then added to the blots, and they were incubated for an additional 1 h. We washed the blots four times with PBS for a total of 30 min and then added a secondary goat anti-mouse horseradish peroxidase-conjugated antibody (1:1,000) in 5% milk-PBS for 1 h. The blots were then washed four times with PBS for a total of 2 h. A chemiluminescence kit (Pierce) was used as substrate for the horseradish peroxidase conjugate, and the image of the blot was recorded on scientific imaging film (Kodak).

Tissue culture cells. Unless otherwise noted, assays involving tissue culture cells were conducted in RP10 medium (RPMI 1640, 10% fetal calf serum, L-glutamine, HEPES, 50 µM 2-mercaptoethanol). Tissue culture cells were grown in RP10 medium containing penicillin (50 U/ml) and streptomycin (50 µg/ml). RAW309.1 macrophages and 1308.1 epithelial cells were used to present antigen in CD8⁺-T-cell activity assays. The OVA₂₅₇₋₂₆₄-specific CD8⁺-T-cell hybridoma B3Z served as effectors (15). In CD4⁺-T-cell assays, bone marrow-derived macrophages isolated from C57BL/6 mice (Jackson Laboratories) were used as professional antigen-presenting cells. Bone marrow-derived macrophage isolation has been described previously (2). In these assays, the OVA₃₂₃₋₃₃₉-specific CD4⁺-T-cell hybridoma BO-97.11 was used as the effector (9). BO-97.11

cells were grown in Dulbecco's modified Eagle medium, 10% fetal calf serum, 10 mM HEPES, 50 U of penicillin/ml, and 50 µg of streptomycin/ml.

CD4⁺-T-cell activity assay. Cells infected with the serovar Typhimurium strains expressing the F8M4-tagged proteins were assayed to determine the expression and processing of the OVA₃₂₃₋₃₃₉ epitope. The antigen-presenting cells used in this assay were bone marrow-derived macrophages isolated from C57BL/6 mice. Surface expression of OVA₃₂₃₋₃₃₉ was detected by using the specific CD4⁺-T-cell hybridoma BO-97.11 (9). Serovar Typhimurium strains were grown overnight in 1 ml of LB medium containing 10 mM IPTG at 37°C with agitation. Two microliters of live bacteria and 5 × 10⁴ C57BL/6 bone marrow-derived macrophages were added to a flat-bottom 96-well plate in a total volume of 100 µl and centrifuged at 1,000 × *g* for 5 min. Bacteria and macrophages were incubated at 37°C in 7% CO₂ for 3 h, followed by the addition of 5 × 10⁵ BO-97.11 cells in 100 µl of Dulbecco's modified Eagle medium with 10% fetal calf serum and 50 µg of gentamicin/ml. After the addition of the BO-97.11 cells, the plate was incubated for 16 h at 37°C in 7% CO₂. The assay plate was then centrifuged for 10 min, and medium from each well was tested for interleukin 2 (IL-2) by using an IL-2 enzyme-linked immunosorbent assay kit (Pierce Endogen) according to the manufacturer's instruction.

CD8⁺-T-cell activity assay. CD8⁺-T-cell activity was used to identify *C. trachomatis* proteins which are translocated from serovar Typhimurium into the cytosol of eukaryotic cells. B3Z is an OVA₂₅₇₋₂₆₄-specific CD8⁺-T-cell hybridoma that expresses the β-galactosidase gene under the control of the NK-AT element of an IL-2 enhancer (11). Thus, increased β-galactosidase activity of B3Z cells correlates with stimulation of these OVA₂₅₇₋₂₆₄-specific CD8⁺ T cells.

Bacteria were grown overnight in 1 ml of LB medium at 37°C without agitation in 1.5-ml Eppendorf tubes. The bacterial culture was subcultured (1:10) into 1 ml of fresh LB medium and again incubated at 37°C overnight without agitation in 1.5-ml Eppendorf tubes. Two microliters of the bacterial culture was added to 5 × 10⁴ RAW309.1 or 1308.1 cells in 100 µl of RP10 medium without antibiotics in a round-bottom 96-well plate. 1308.1 cells were seeded 6 h prior to the addition of the bacteria to allow the cells to adhere.

The bacteria and host cells were centrifuged at 1,000 × *g* for 5 min at room temperature to synchronize the infection and then incubated at 37°C in 7% CO₂ for 1 h. B3Z cells (5 × 10⁴) in 100 µl of RP10 medium containing gentamicin (50 µg/ml) were then added to each well. The infected host cells and B3Z cells were coinoculated at 37°C in 7% CO₂. After overnight incubation, 160 µl of medium was removed from each well and discarded. We then added 100 µl of Z buffer (9 mg of chlorophenol red-β-D-galactopyranoside [Calbiochem], 900 µl of 1 M MgCl₂, 124 µl of NP-40, 180 µl of β-mercaptoethanol, 98 ml of PBS) to each well. Each assay was performed simultaneously in three to six identical wells. The optical density at 595 nm (OD₅₉₅) was measured after 4 to 8 h and normalized to the mean of the β-galactosidase measurements obtained from host cells infected with the serovar Typhimurium expressing the negative control LacZ-F8M4. *P* values were calculated by using Student's *t* test.

RESULTS

F8M4 plasmids allow multiple tags to be fused to target proteins. In order to tag proteins with specific peptide sequences that could be used in different expression and translocation screens, we constructed a DNA fragment designated F8M4. The F8M4 construct (Fig. 1A) encodes several peptide motifs and epitopes, including the FIAsh sequence, and the OVA₂₅₇₋₂₆₄, *c-myc*, and OVA₃₂₃₋₃₃₉ epitopes. The FIAsh motif encodes a CCXXCC peptide sequence to which the Lumio reagent (Invitrogen) binds. When the FIAsh motif binds the Lumio reagent, a nonfluorescent fluorescein derivative which can cross eukaryotic cell membranes of live cells, the FIAsh-bound Lumio reagent then fluoresces (6). The FIAsh motif is followed by the OVA₂₅₇₋₂₆₄ CD8⁺-T-cell epitope. If OVA₂₅₇₋₂₆₄ is present in the cytoplasm of host cells, it will target those cells for recognition by OVA₂₅₇₋₂₆₄-specific CD8⁺ T cells (14). The *c-myc* epitope is recognized by anti-*c-myc* antibody, which can be used to identify and localize F8M4-tagged proteins or to purify these proteins by immunoprecipitation. Lastly, the OVA₃₂₃₋₃₃₉ peptide sequence encodes an epitope which binds MHC-II molecules (9). When this epitope

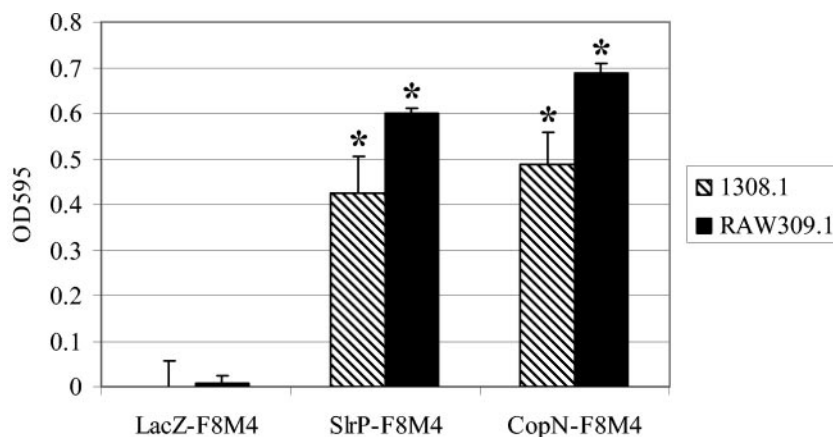


FIG. 3. Activity of OVA₂₅₇₋₂₆₄-specific CD8⁺ T cells. An OVA₂₅₇₋₂₆₄-specific CD8⁺-T-cell hybridoma (B3Z) was added to host cells infected with each serovar Typhimurium strain expressing a F8M4-tagged protein. OD₅₉₅ corresponds to the activity of B3Z. Serovar Typhimurium strains expressing LacZ-F8M4, SlrP-F8M4, or CopN-F8M4 were used to infect RAW309.1 macrophages (filled bars) or 1308.1 epithelial cells (hatched bars). *, $P < 0.0004$.

tag is processed and presented by professional antigen-presenting cells, such as macrophages and dendritic cells, they can activate OVA₃₂₃₋₃₃₉-specific CD4⁺ T cells. These peptide motifs and epitopes are flanked by inverted repeat sequences which are recognized by the Tn5 transposase, allowing the transposition of the DNA between the Tn5 inverted repeat ends. We added the HA epitope and a selectable antibiotic resistance gene (*cat*) to the F8M4 element to generate TnF8M4. We have demonstrated that the TnF8M4 transposon can be randomly inserted onto a plasmid encoding a protein of interest by using EZ::TN in vitro transposition (data not shown). Once proteins of interest are tagged with F8M4, multiple screening tools can be used to study their expression and subcellular localization within host cells.

When fused to a known *Salmonella* type III secreted protein, F8M4 is translocated into the host cell cytoplasm. To show that *Salmonella* can be used to translocate proteins tagged with F8M4 into the host cell cytosol, the serovar Typhimurium protein SlrP was fused to F8M4 as a positive control. The SlrP protein is translocated at a high level during infection of both macrophages and epithelial cells via the *Salmonella* pathogenicity island 1 (SPI-1) and SPI-2 type III secretion systems (13). The F8M4 tag contains the OVA₂₅₇₋₂₆₄ CD8⁺-T-cell epitope. If a protein tagged with this epitope is translocated from serovar Typhimurium into the cytoplasm of a mammalian cell, the epitope will be processed in the cells and target the cells for recognition by OVA₂₅₇₋₂₆₄-specific CD8⁺ T cells. We tested whether CD8⁺ T cells could detect translocation of the SlrP-F8M4 protein in cells infected with serovar Typhimurium expressing the tagged protein. RAW309.1 macrophages or 1308.1 epithelial cells infected with serovar Typhimurium expressing SlrP-F8M4 stimulated significant CD8⁺-T-cell activity (Fig. 3), suggesting that SlrP-F8M4 protein was translocated by serovar Typhimurium into the host cell cytoplasm.

As a negative control, the LacZ protein was tagged with F8M4. The LacZ protein cannot be translocated by *Salmonella* and remains within the bacterial cytoplasm (13). When RAW309.1 or 1308.1 cells were infected with serovar Typhimurium expressing LacZ-F8M4 or the isogenic serovar Typhi-

murium control strain lacking the *lacZ*-F8M4 plasmid, we found that neither strain stimulated CD8⁺-T-cell activity (data not shown). This result demonstrated that a F8M4-tagged protein retained in the bacterial cytoplasm during host cell infection failed to stimulate CD8⁺ T cells in our assay.

Because the differences between our positive and negative controls in the CD8⁺-T-cell activity assays might have resulted from differences in the levels of SlrP and LacZ protein expressed by serovar Typhimurium, we compared the levels of expression of F8M4-tagged SlrP and LacZ protein. Using immunoblot analysis probed with anti-c-myc antibody, we found that both the F8M4-tagged LacZ' and SlrP' proteins were abundantly expressed in serovar Typhimurium when grown in culture. We used another assay to compare the expressions of the two F8M4-tagged proteins during infection of professional antigen-presenting cells. Bone marrow macrophages were infected with serovar Typhimurium expressing the F8M4-tagged LacZ or SlrP protein. When these tagged proteins are processed in professional antigen-presenting cells, such as macrophages, the OVA₃₂₃₋₃₃₉ epitope is presented on the cell surface and can activate the OVA₃₂₃₋₃₃₉-specific CD4⁺-T-cell hybridoma BO-97.11. We found that there was an increase in IL-2 production by BO-97.11 cells when they were exposed to professional antigen-presenting cells infected with serovar Typhimurium expressing either F8M4-tagged SlrP or F8M4-tagged LacZ (a 29% increase in IL-2 for F8M4-tagged SlrP [$P < 0.003$] and a 44% increase in IL-2 for F8M4-tagged LacZ [$P < 0.036$] compared to results with serovar Typhimurium without the F8M4 vector). This finding suggested that the serovar Typhimurium expressed sufficient levels of the F8M4-tagged proteins for the OVA₃₂₃₋₃₃₉ epitope to be processed and presented in the context of MHC-II molecules during infection of bone marrow macrophages. The results of these CD4⁺-T-cell activity assays combined with the immunoblot analysis show that serovar Typhimurium expressed similarly high levels of SlrP-F8M4 and LacZ-F8M4 protein when the bacteria were grown in culture or when they were used to infect bone marrow macrophages. Therefore, although F8M4-tagged SlrP and LacZ proteins were expressed at the same high levels, only the

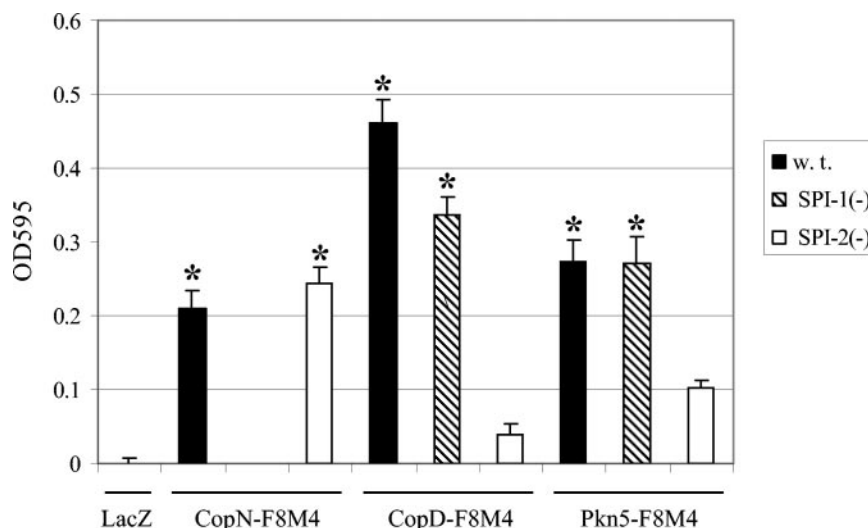


FIG. 4. Activity of OVA₂₅₇₋₂₆₄-specific CD8⁺ T cells. An OVA₂₅₇₋₂₆₄-specific CD8⁺-T-cell hybridoma (B3Z) was added to host cells infected with serovar Typhimurium strains expressing F8M4-tagged proteins. OD₅₉₅ corresponds to the activity of B3Z. RAW309.1 macrophages were infected with serovar Typhimurium strains expressing LacZ-F8M4, CopN-F8M4, CopD-F8M4, or Pkn5-F8M4. F8M4-tagged proteins were expressed in wild-type (w.t.) serovar Typhimurium (filled bars), SPI-1 type III secretion system-deficient serovar Typhimurium (hatched bars), or SPI-2 type III secretion system-deficient serovar Typhimurium (open bars). *, $P < 0.0004$.

type III secreted SlrP-F8M4 protein was translocated by serovar Typhimurium into the host cell cytoplasm, resulting in the stimulation of CD8⁺ T cells.

The *C. trachomatis* CopN protein is translocated by the *Salmonella* SPI-1-encoded type III secretion system. The *C. trachomatis* protein CopN was tested to confirm that CD8⁺-T-cell reactivity could be used to identify a F8M4-tagged *C. trachomatis* type III secreted effector. Previously, it was shown by using immunofluorescence microscopy that an epitope-tagged CopN protein fusion can be translocated into the eukaryotic cytoplasm by *Y. enterocolitica* (3). To determine whether F8M4-tagged CopN can also be translocated by serovar Typhimurium, we cloned the entire open reading frame of the *C. trachomatis* *copN* gene upstream of the F8M4 tag (Fig. 2C). We then infected RAW309.1 macrophages or 1308.1 epithelial cells with serovar Typhimurium containing the *copN*-F8M4 plasmid and tested for CopN-F8M4 translocation by using CD8⁺-T-cell activity assays. The infected mammalian cells stimulated significant CD8⁺-T-cell activity (Fig. 3). Activity of the CD8⁺ T cells strongly suggested that CopN was translocated by *Salmonella*. Although we detected CopN-F8M4 protein when CopN-F8M4 was expressed in *Escherichia coli*, CopN-F8M4 expression in serovar Typhimurium was too low to detect by anti-*c-myc* antibody immunoblotting. Despite the very low expression of CopN-F8M4, the CD8⁺-T-cell activity assay was sensitive enough to detect translocation of CopN-F8M4 by serovar Typhimurium.

Salmonella expresses two type III secretion systems, which are each encoded on a different SPI, designated SPI-1 and SPI-2 (8). To determine which system was responsible for the translocation of CopN by *Salmonella*, we transformed the *copN*-F8M4 plasmid into serovar Typhimurium mutants which lacked one of the *Salmonella* type III secretion systems. Although RAW309.1 cells infected with the serovar Typhimurium strain lacking the SPI-2-encoded type III secretion

system were able to stimulate CD8⁺ T cells, macrophages infected with CopN-F8M4-expressing serovar Typhimurium which lacked the SPI-1-encoded type III secretion system were not able to activate CD8⁺ T cells (Fig. 4). This finding suggested that CopN was likely translocated into host cells by the SPI-1-encoded type III secretion system of *Salmonella*.

The *C. trachomatis* proteins CopD and Pkn5 are translocated into host cells by the *Salmonella* SPI-2 type III secretion system. Two other candidate *C. trachomatis* proteins were also examined to determine whether they could be translocated into the cytosol of host cells by serovar Typhimurium. Although the *Chlamydia* CopD and Pkn5 proteins have been postulated to be type III secreted effectors (12), there has been no experimental evidence with *Chlamydia* or with heterologous systems to show that CopD and Pkn5 are substrates of type III secretion. We tagged CopD and Pkn5 with the F8M4 element and expressed them in serovar Typhimurium. We found that RAW309.1 macrophages infected with serovar Typhimurium expressing either CopD-F8M4 or Pkn5-F8M4 stimulated significant CD8⁺-T-cell activity (Fig. 4). This T-cell activity suggested that CopD and Pkn5 proteins were translocated by serovar Typhimurium into the host cell cytoplasm.

To further demonstrate that these proteins were translocated via a *Salmonella* type III secretion system, we transformed the *copD*- and *pkn5*-F8M4 plasmids into serovar Typhimurium mutants which lacked the SPI-1- or SPI-2-encoded type III secretion system. Host cells infected with SPI-1-deficient serovar Typhimurium expressing CopD or Pkn5 were able to stimulate CD8⁺ T cells (Fig. 4). However, host cells infected with SPI-2-deficient serovar Typhimurium expressing CopD or Pkn5 stimulated substantially less CD8⁺-T-cell activity (Fig. 4). Therefore, unlike the CopN protein, the *C. trachomatis* CopD and Pkn5 proteins were translocated into eukaryotic cells by serovar Typhimurium in a SPI-2-encoded type III secretion-dependent manner.

DISCUSSION

We have developed a technique to identify *Chlamydia* proteins that serve as substrates for type III secretion. By tagging *C. trachomatis* proteins with multiple peptide motifs and epitopes, we can use the tools of different arms of the adaptive immune system to screen for proteins which are expressed in host cells during infection. Although the F8M4 system allows for the investigation of antibody and CD4⁺-T-cell responses, we have focused on the use of this system to identify *Chlamydia* proteins that can be translocated into the host cell cytosol via a type III secretion system. Using CD8⁺ T cells as a probe, we have confirmed that CopN, a *C. trachomatis* protein shown to be translocated by *Yersinia* (3), can also be translocated by the *Salmonella* SPI-1 type III secretion system. Additionally, we have demonstrated that the *Salmonella* SPI-2 type III secretion system can translocate two other *C. trachomatis* proteins, Pkn5 and CopD, into host cells. Although *C. trachomatis* appears to have only a single type III secretion system, the secretion of some effectors by the *Salmonella* SPI-1 system and of others by the SPI-2 system may provide information about what is required to target proteins to the *C. trachomatis* type III secretion system.

Pkn5 was originally identified during the sequencing of the *C. trachomatis* genome. The *pkn5* gene is encoded on a *Chlamydia* type III secretion system subcluster, where it is flanked by genes encoding putative structural components of the secretion apparatus (20). Pkn5 was annotated as a putative serine/threonine kinase (18). However, Pkn5 lacks important structural residues which are necessary in other serine/threonine kinases for activity (22), and purified Pkn5 does not exhibit protein kinase activity in vitro (22). Although Pkn5 appears to be transcriptionally active in *Chlamydia*, its role in *C. trachomatis* infection is not known.

The other putative type III secreted effector we have examined in this study is CopD. The CopD protein is abundant in the elementary body form of *Chlamydia* (4, 16). Because of its abundance, CopD may play an important role in the CD4⁺-T-cell response to *C. trachomatis* infection. Indeed, a human CD4⁺-T-cell clone has been shown to be specific to CopD (5), suggesting that CopD is a CD4⁺-T-cell antigen. The CopD protein has no recognizable secretion sequence, and its size and hydrophobicity profile are similar to those of *Yersinia* YopD (19). In *Yersinia*, YopD, in complex with YopB, is believed to form a porin-like structure in the host cell membrane which facilitates the translocation of other type III secreted substrates (1). Although there is no obvious sequence homology between CopD and YopD (19), it has been hypothesized that CopD may play a similar role in *Chlamydia* type III secretion.

We have shown that the CopD, Pkn5, and CopN proteins can be translocated into host cell cytoplasm by serovar Typhimurium. If these proteins have access to the host cell cytoplasm during *C. trachomatis* infection, they might elicit CD8⁺-T-cell responses in infected individuals. We are currently studying these translocated *Chlamydia* proteins to determine if these proteins contain endogenous CD8⁺-T-cell epitopes that prime CD8⁺-T-cell responses during *C. trachomatis* infection.

In parallel with the work presented here, we have conducted some experiments using an adenylate cyclase (CyaA') reporter

system (17). Although we observed the expression of candidate *Chlamydia* type III secreted substrates fused to the CyaA' reporter in serovar Typhimurium, we were unable to detect translocation of any *Chlamydia* CyaA' protein fusion into host cells (T. D. Ho, W. Lencer, and M. N. Starnbach, unpublished data.). Because CopN has previously been shown to be translocated, we concluded that the CyaA' reporter system was less sensitive than CD8⁺ T cells as a detection tool. Therefore, we focused on using CD8⁺ T cells as a tool to identify translocated *Chlamydia* proteins.

CD8⁺-T-cell reactivity could be used to comprehensively test each *C. trachomatis* protein in the genome to see whether it is able to serve as a substrate for type III secretion in *Salmonella*. Because the C-terminal F8M4 tag is less than 100 amino acids in size, it is less likely to interfere with translocation than other, larger tags. It also does not have to retain enzymatic activity, a drawback of other reporter systems. The CD8⁺-T-cell assay itself is technically simple and is performed in a 96-well format; both of these characteristics become important when screenings are conducted on a large scale. A library of F8M4-tagged proteins can be generated by cloning each *C. trachomatis* gene onto the F8M4 plasmid or by transposing TnF8M4 onto existing *C. trachomatis* genomic libraries. By using CD8⁺-T-cell activity as a readout, these libraries then can be screened for candidate type III secreted proteins.

Although we believe that this method will be useful in identifying many proteins that are translocated during *Chlamydia* development, there are limitations inherent in this technique. While the CD8⁺-T-cell activity assay will be sensitive enough to detect many translocated *Chlamydia* proteins, the assay will probably not identify all translocated *Chlamydia* proteins, especially those which are poorly expressed in serovar Typhimurium and those which are not similar enough to *Salmonella* effectors to be translocated by the heterologous serovar Typhimurium type III secretion systems. It is also possible that some *Chlamydia* proteins secreted by serovar Typhimurium may not be translocated by the *Chlamydia* type III secretion system. We envision that when techniques are developed to introduce DNA into *Chlamydia*, we could saturate the *C. trachomatis* genome with the TnF8M4 transposon. The resulting *C. trachomatis* library could be screened with both cell culture and experimental animals for the response of antibody, CD4⁺ T cells, and CD8⁺ T cells. This screening would allow for a comprehensive analysis of which arms of the adaptive immune system are responding to each protein at various stages of development in vitro and at various stages of infection in vivo. Until such genetic tools are developed, we are restricted to testing for translocation of *Chlamydia* proteins by using heterologous type III secretion systems. Our present study demonstrates that CD8⁺-T-cell reactivity can be used to test candidate *Chlamydia* proteins that are translocated via a type III secretion system.

Our method of detecting translocated proteins is not limited to the study of *Chlamydia* proteins. The CD8⁺-T-cell activity assay can be used to identify proteins in many different bacterial pathogens that have access to the host cell cytoplasm. By generating a genomic library of F8M4-tagged proteins, a comprehensive study of a genetically recalcitrant bacterium can be conducted in the same way we have described for *C. trachomatis*. In organisms for which genetic tools are available, the

TnF8M4 transposon can be used to generate a library of F8M4-tagged proteins. For instance, one could generate a serovar Typhimurium library of F8M4-tagged proteins by using the TnF8M4 transposable element to identify as-yet-unidentified *Salmonella* type III secretion substrates. The F8M4 element allows us to use of a number of tools to identify candidate virulence proteins and to understand the adaptive immune responses elicited by those proteins.

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