Published in final edited form as:

Microb Pathog. 2008 ; 45(5-6): 435–440. doi:10.1016/j.micpath.2008.10.002.

Induction of Type III Secretion by Cell-Free *Chlamydia trachomatis* Elementary Bodies

Wendy P. Jamison and Ted Hackstadt*

Host-Parasite Interactions Section, Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, MT 59840

Abstract

Chlamydiae secrete type III effector proteins at two distinct stages of their developmental cycle. Elementary bodies (EBs) secrete at least one pre-formed effector protein, Tarp, across the host plasma membrane from an extracellular location. Once internalized, a set of newly transcribed proteins are secreted to modify the inclusion membrane. In an effort to better understand the triggers for chlamydial type III secretion and develop means to identify new effectors, we investigated various inducers of T3SS in other Gram-negative bacterial systems to determine if they were able to activate chlamydial type III secretion from EBs using Tarp secretion as an indicator of activation. Chlamydial EBs are induced to secrete Tarp by exposure to FBS, BSA, or sphingolipid and cholesterol-rich liposomes (SCRL). The induction by FBS and BSA, but not SCRL, is enhanced in the presence of the calcium-chelator, EGTA. This secretion was temperature dependent and inhibited by paraformaldehyde fixation of the EBs.

Keywords

Chlamydia trachomatis; type III secretion; actin; endocytosis

1. Introduction

Chlamydia trachomatis is an obligate intracellular bacterium that is the causative agent of several distinct human diseases. There are over 15 serologically defined variants, or serovars, of *C. trachomatis*. Serovars A–C are associated with endemic, blinding trachoma, Serovars D – K with sexually transmitted diseases, and serovars L1, L2, and L3 with lymphogranuloma venereum, a more systemic disease that is also sexually transmitted [1].

Chlamydiae have a unique biphasic developmental cycle consisting of cell types adapted for extracellular survival and intracellular growth. The developmental cycle of *Chlamydia* begins when the metabolically inert, environmentally stable infectious particles, termed elementary bodies (EBs), invade target host cells. Once inside the host cell, the EBs differentiate into vegetative, non-infectious forms, termed reticulate bodies (RBs), which replicate within the membrane-bound parasitophorous vacuole called an inclusion [2].

Type III secretion systems (T3SSs) are known virulence determinants used by pathogenic Gram-negative bacteria to modulate the host cell environment by translocating bacterial effector proteins into the host cell [3]. The supramolecular structure of the T3SS apparatus resembles a needle complex that spans the bacterial and host cell membranes [4]. The components of the secretion apparatus are generally conserved, however, the specific effector

*Corresponding author, Phone (406)-363-9308, Fax (406)-363-9253, e-mail ted_hackstadt@nih.gov.

proteins delivered are typically unique to a species. The functions of these injected effectors are multitude. Many affect cellular signal transduction pathways to modulate cytoskeletal dynamics [5,6], inhibit cytokine production [7,8], inhibit, or promote apoptosis [7] and other cellular pathways to promote pathogen survival [9].

Chlamydiae encode the components of a type III secretion apparatus yet the full complement of effector proteins secreted remains unknown [10]. In contrast to most bacterial pathogens, the chlamydial T3SS genes occur in clusters throughout the genome, rather than being concentrated on pathogenicity islands [11]. While the majority of the T3SS genes are not expressed until middle to late stage of the development cycle [12], proteomic analyses indicate that the essential components of the secretory apparatus are present on EBs [4]. Structurally, projections have been observed on the surface of both EBs and RBs in electron micrographs that may correspond to the T3SS apparatus [13,14]. Chlamydiae appear to use type III secretion during at least two distinct stages in their development. During entry, extracellular, plasma membrane-associated EBs secrete a pre-existing effector protein, termed Tarp, for translocated actin recruiting protein, across the host cytoplasmic membrane where *C. trachomatis* Tarp is tyrosine-phosphorylated and functions in the nucleation of actin required for entry [15,16]. Once internalized, de novo synthesized effector proteins are also secreted across the inclusion membrane such that they are exposed to the cytosol [17,18]. Chlamydiae do not have two complete sets of secretion apparatus, but certain individual components appear to be duplicated, thus the T3SSs operating at the distinct locations may be structurally distinct [19,20]. Due to limited genetic tools available in *Chlamydia*, the putative T3SS effectors identified thus far have been confirmed using heterologous expression systems for T3SS effectors [10,12,21–24].

Many intracellular and epicellular pathogens secrete type III effectors that alter cytoskeletal dynamics [9]. Typically, multiple effector proteins are translocated upon contact. The only known effector secreted by chlamydiae immediately upon contact with host cells is Tarp [15]. It is likely that other, pre-existing effector molecules are also secreted upon contact with the host cell. The environmental signals that trigger secretion are poorly understood but there are many examples of induction of T3SS in the absence of host cells. Studies of Gram-negative organisms, including *Shigella*, *Salmonella*, *Pseudomonas*, *Yersinia*, and *Campylobacter*, have shown the following conditions are able to trigger induction of the T3SSs: temperature [25], low calcium [25], fetal bovine serum (FBS) [26], bovine serum albumin (BSA) [27], glutamate [27], congo red [28], and liposomes [29]. To date, the induction trigger of the T3SS in *Chlamydia* has not been identified. Because of the limited opportunities for molecular genetic analysis of T3S in chlamydiae, we investigated several known inducers of T3SSs in an attempt to find some that induce secretion from EBs. Several *in vitro* inducers of T3SSs functioned on EBs, and may provide a means to identify or confirm additional putative chlamydial type III effectors.

2. Results

2.1. Induction of chlamydial EB type III secretion

It was first necessary to determine a buffer in which the EBs would be stable with a minimum background release of proteins. We analyzed a variety of buffers, including 220 mM sucrose-50 mM sodium phosphate-5 mM glutamate, pH 7.4 (SPG), Hank's balanced salt solution (HBSS), 50 mM NaPO₄, 150 NaCl, pH 7.4 (PBS), 50 mM potassium acetate, pH 4.8, 0.25 M sucrose, and water. As shown in Fig. 1, 50 mM potassium acetate elicited a minimal release of protein as detected by silver staining. Immunoblotting with an α -Tarp antibody detected little to no Tarp released after exposure to the potassium acetate buffer or SPG. Finally, immunoblotting with an α -EB antibody indicated little release of EB antigens or lysis of EBs. The potassium acetate buffer was therefore used for all future T3S experiments.

Induction conditions for the T3SS of chlamydial EBs were tested using inducers that had been previously described in other Gram-negative bacterial pathogens and using Tarp release as an indicator of T3S induction in EBs. Several compounds known to induce T3S in various bacteria were prepared in 50 mM potassium acetate, pH 4.8 including: 3% fetal bovine serum (FBS), 0.5% bovine serum albumin (BSA) and 200 μ g sphingolipid and cholesterol-rich liposomes (SCRLs), 135 μ M glutamate, and 10 μ M Congo red. Potassium acetate buffer was used as a negative control and resulted in minimal protein detection with the α -Tarp antibody as shown in Fig. 2. 3% FBS, 0.5% BSA and 200 μ g liposome treatment of EBs all resulted in secretion of Tarp. Glutamate and Congo red treatment did not result in Tarp detection above background levels. Probing for chlamydial protein with an anti-EB antiserum indicated that Tarp secretion was specific and not due to lysis of EBs under the induction conditions.

The calcium chelator, EGTA, has been reported to enhance T3S *in vitro* [30–32]. EGTA was therefore added to a final concentration of 10 mM in potassium acetate buffer in the presence or absence of the various inducers described above. Fig. 3 demonstrates that EGTA had very little effect on the amount of Tarp secreted when the EBs were suspended in potassium acetate buffer. However, the addition of 10 mM EGTA to either FBS or BSA resulted in a dramatic increase in the amount of Tarp secreted. Similar increases were not observed with the SCRLs when EGTA was present (data not shown).

2.2. Temperature dependency of the T3SS in chlamydial EBs

Tarp secretion by EBs in association with HeLa cells is temperature dependent [15]. The temperature dependency of the T3SS of chlamydial EBs *in vitro* was determined. We followed the same induction protocol, however, we incubated EBs at either 4°C or 37°C for 30 minutes. As shown in Fig. 4, the amount of Tarp detected at 37°C after exposure to SCRLs was much greater than at 4°C.

2.3. Effect of induction on infectivity of chlamydial EBs

To determine if *in vitro* induction of the T3SS had an adverse effect upon the infectivity of chlamydiae, EBs were exposed to the various inducers and the titers (IFUs) were determined, as shown in Table 1. No reduction in the infectivity of the EBs was observed after exposure to any of the experimental conditions. A considerable amount of Tarp remains associated with EBs after induction (Fig. 5), thus triggering secretion *in vitro* does not apparently deplete Tarp stores and the EBs remain infectious.

2.4. Effect of fixation on induction of T3S and entry of chlamydial EBs

To determine the effect of fixation on attachment and internalization of *Chlamydia trachomatis* L2 EBs, the EBs were fixed for 30 min in 4% paraformaldehyde, 2.5% glutaraldehyde, or mock treated, and Tarp secretion *in vitro* as well as the effect on interactions with HeLa cells were determined. As shown in Fig. 6A, Tarp was not detected in the supernatant of induced chlamydial EBs previously fixed with 4% paraformaldehyde. Control unfixed EB's secreted Tarp when induced with SCRLs. Corresponding results were obtained when fixed and unfixed chlamydial EBs were analyzed by immunofluorescence for Tarp phosphorylation and in an invasion assay (Fig. 6B). Paraformaldehyde-fixed chlamydial EBs attached to HeLa cells but were not internalized. This was in contrast to the control, unfixed EBs of which 73.2 % were internalized. The presence of phosphorylated Tarp was also examined as an indicator of Tarp secretion. Unlike the control EBs, no phosphorylated Tarp was associated with paraformaldehyde-fixed EBs attached to HeLa cells.

3. DISCUSSION

Most Gram-negative pathogens secrete a battery of effector proteins in response to the appropriate stimulatory signal. Typically, contact of a bacterium with the host cell activates type III secretion although in many cases environmental stimulators of T3S have been identified that trigger secretion in the absence of host cells [25–29]. In an effort to better understand induction of T3S by chlamydiae, we have screened several potential inducers for stimulation of Tarp secretion by cell-free EBs. Fetal bovine serum, albumin, and SCRLs activated Tarp secretion in a temperature dependent fashion. Here we demonstrate a novel and biologically relevant method to induce the T3SS of chlamydial EBs in the absence of cell contact. Identification of those environmental cues triggering chlamydial T3S may suggest novel means to interrupt a critical process in the interactions of chlamydiae with eukaryotic host cells.

With no genetic manipulation tools available, we pursued developing a way to investigate secreted effector proteins of chlamydial EBs upon induction of the T3SS. The T3SS on chlamydial EBs appears to be preassembled [10] and at least one effector is presynthesized for secretion upon contact with the host cell. [33–35]. A variety of environmental conditions have been identified as capable of inducing the T3SS in Gram-negative bacteria in a cell-free environment [25–29]. We tested previously characterized conditions for induction of the chlamydial T3SS. Our results indicate that both FBS and BSA induce secretion of Tarp from EBs, while congo red and glutamate did not. SCRLs also resulted in Tarp secretion. As with *Shigella* [29], this may suggest involvement of ordered lipid domains in the induction of the chlamydial T3SS. Indeed, lipid rafts have been implicated in the internalization of some chlamydial strains [36] although these findings have been questioned [37].

EGTA has also been used to enhance T3SS in Gram-negative bacteria due to its functionality as a calcium chelator [30,31]. The addition of EGTA greatly increased the amount of secreted Tarp when the EBs were induced with either FBS or BSA. EGTA alone did not result in a similar increase in the amount of Tarp detected in the supernatant. Interestingly, the same increase in Tarp secretion was not detected when EGTA was added to the SCRLs (data not shown). In any event, the amount of Tarp secreted in these *in vitro* assays appears to be a relatively small proportion of the total Tarp in EBs and triggering of secretion did not significantly reduce the subsequent infectivity of EBs. Of course the amount of Tarp secreted upon contact of EBs with susceptible host cells is also unknown and may represent only a small percentage of the total complement of Tarp in EBs.

EBs have a pre-formed type III secretion apparatus but display no known metabolic or transcriptional activity [2,10]. Tarp is pre-existing in EBs in an unphosphorylated state and not exposed on the surface of EBs. Its translocation occurs upon contact with host cells even in the presence of inhibitors of bacterial transcription or translation [15]. Thus chlamydial T3S may be activated to secrete in the absence of gene expression. Despite the lack of measurable metabolic activity, EBs have an unusually high ATP content of about 40 mM [38] that is presumably necessary for early events including activation of T3S. Incubation at 4°C inhibits Tarp secretion either into cells [15] or by cell-free inducers of T3S. Paraformaldehyde fixation of chlamydial EBs inhibited induction of Tarp secretion by SCRL as well as by cellular contact but did not appreciably reduce attachment. Invasion assays corroborated these results as paraformaldehyde-fixed EBs did not become internalized. The data support the necessity for Tarp secretion from EBs for entry, but not for attachment.

We have demonstrated here that chlamydial EBs can be induced to secrete T3S effectors in the absence of host cell contact. The ability to induce secretion using SCRLs allows for a protein-free induction which may permit identification of additional T3S effector proteins

secreted at the early stages of infection. Type III secretion is an important virulence determinant of many Gram negative bacteria including chlamydiae and an attractive target for interruption of pathogen development. Small molecule inhibitors of T3S have been identified and shown to inhibit T3S and chlamydial development [39,40]. An improved understanding of the signals mediating chlamydial type III secretion as well as the complement of effectors secreted should provide for additional targets to inhibit chlamydial pathogenesis.

4. Materials and Methods

4.1. Organisms and cell culture

C. trachomatis L2 (LGV-434) was grown in HeLa 229 cells as previously described [41]. EBs were purified by Renografin (E. R. Squibb and Sons, Princeton, NJ) density gradient centrifugation [41]. Inclusion forming units (IFUs) were determined as previously described [42].

4.2. SDS-PAGE and immunoblotting

Proteins were separated on 9% polyacrylamide gels [43]. Proteins were then silver stained according to the manufacturer's protocol using the SilverQuest silver staining kit (Invitrogen, Carlsbad, CA). Immunoblotting was performed by transferring the proteins to a 0.2 μ m Protran nitrocellulose membrane (PerkinElmer, Boston, MA). Immunoblots were developed using Super Signal West Pico chemiluminescence reagent (Pierce Biotechnology, Rockford, IL). Polyclonal rabbit antibodies to Tarp were previously described [15]. Polyclonal rabbit antibodies to *C. trachomatis* L2 elementary bodies have also been described.

4.3. Liposome preparation

Lipids were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Lipids used were: phosphatidylcholine:sphingomyelin:cerebrosides:cholesterol, 2:1:1:2 (mole ration). Large multilamellar liposomes were prepared according to a previously described protocol [44]. Briefly, the chloroform solution of each lipid was mixed, dried under nitrogen gas, then freeze-dried. The liposomes were resuspended in 50 mM potassium acetate buffer, pH 4.8 at 85°C. Liposomes were then vortexed for 30 seconds, incubated for 30 minutes at 85°C, vortexed for 45 seconds, and incubated for 15 minutes at 85°C. Finally, liposomes were allowed to cool at room temperature for a minimum of 30 minutes.

4.4. Induction protocol

C. trachomatis L2 EBs stored at -80°C in SPG buffer were thawed and aliquots of 500 μ l (approx. $8 \times 10^9 - 1 \times 10^{10}$ Inclusion Forming Units) made into microcentrifuge tubes. EBs were pelleted by centrifugation at 15,000 RPM in a TOMY MR-150 centrifuge at 4°C for five minutes. The supernatant was discarded and the EBs were resuspended in 250 μ l 50 mM potassium acetate buffer, pH 4.8. The EBs were pelleted again and the supernatant was discarded. EBs were then resuspended in 55 μ l of the appropriate experimental solution and incubated in a 37°C incubator for thirty minutes. After the incubation, the EBs were pelleted, the supernatant transferred to a new microcentrifuge tube, and the EB pellets were discarded. The supernatant was centrifuged again, and 48 μ l of the supernatant was transferred to a new microcentrifuge tube containing 12 μ l sample buffer. 23 μ l of the supernatant and sample buffer solution was loaded onto each gel for analysis.

4.5. Microscopy and internalization assay

To determine the effect of fixation on attachment and internalization of *C. trachomatis* L2 EBs, the EBs were fixed for 30 min in 4% paraformaldehyde in PBS, 2.5% glutaraldehyde in PBS, or mock treated in PBS alone for 30 min at room temperature. The EBs were washed and diluted

in HBSS for plating on HeLa cell monolayers on glass coverslips. The EBs were allowed to attach for 30 min at 4°C and the temperature then shifted to 37°C to permit internalization. The cells were then fixed in 3.7% paraformaldehyde for 15 min, washed in PBS, and blocked with 10% fetal bovine serum for 30 min. Coverslips were incubated with rabbit antibody specific for EBs or mouse monoclonal anti-phosphotyrosine (4G10) for 1 hour. After three washes in PBS, secondary antibodies anti-rabbit IgG-Alexa 488 (Invitrogen, Eugene OR) or anti-mouse IgG-Alexa 594 was added for 45 min. Cover slips were rinsed and mounted in ProLong Gold antifade reagent (Invitrogen). Cells were examined with a Nikon microphot-FXA microscope equipped with phase contrast and epifluorescence optics. Images were obtained using a photometrics coolsnap HQ camera and processed using Adobe Photoshop CS2.

C. trachomatis invasion of HeLa cells was determined essentially as described by Carabeo *et al* [45]. To determine the invasion frequency of fixed *C. trachomatis* L2 EBs, paraformaldehyde or control unfixed EBs were washed and diluted in HBSS for plating on HeLa cell monolayers on glass coverslips. The EBs were allowed to attach for 30 min at 4°C and the temperature then shifted to 37°C to permit internalization. The cells were then fixed in 3.7% paraformaldehyde for 15 min, washed in PBS, and blocked with 10% fetal bovine serum for 30 min. Coverslips were incubated with a monoclonal antibody against the L2 Major Outer Membrane Protein (MOMP) [46] for 1 hr and rinsed with PBS. The cells were then permeabilized with 0.1% Triton X-100 and then incubated with a rabbit anti-L2 antibody for 1 hr. After three washes in PBS, secondary antibodies of anti-rabbit IgG-Alexa 488 (green) (Invitrogen, Eugene OR) or anti-mouse IgG-Alexa 594 (red) were added for 1 hr. Cover slips were rinsed and mounted in ProLong Gold antifade reagent (Invitrogen). The percent of EB invasion per cell was determined as the number of extracellular EBs (red) subtracted from the total number of EBs (green) divided by the total number of EBs (red) multiplied by 100.

Acknowledgements

We thank Janet Sager for excellent technical assistance, the RML Microscopy Unit for confirmation of liposome morphology, Drs. T. Jewett, B. Kleba, and E. Moore for critical reading of the manuscript, and members of the Hackstadt lab for helpful comments and suggestions. This work was supported by the Intramural Research program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

References

- Schachter, J. Infection and disease epidemiology. In: Stephens, RS., editor. Chlamydia; Intracellular Biology, Pathogenesis, and Immunity. Stephens RS: Am Soc Microbiol Press; 1999. p. 139-169.
- Moulder JW. Interaction of chlamydiae and host cells in vitro. Microbiol Rev 1991;55:143-190. [PubMed: 2030670]
- Hueck CJ. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol Mol Biol Rev 1998;62:379-433. [PubMed: 9618447]
- Kubori T, Matsushima Y, Nakamura D, Uralil J, Lara-Tejero M, Sukhan A, Galan JE, Aizawa SI. Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. Science 1998;280:602-605. [PubMed: 9554854]
- Niebuhr K, Giuriato S, Pedron T, Philpott DJ, Gaits F, Sable J, Sheetz MP, Parsot C, Sansonetti PJ, Payraastre B. Conversion of PtdIns(4,5)P(2) into PtdIns(5)P by the *S. flexneri* effector IpgD reorganizes host cell morphology. Embo J 2002;21:5069-5078. [PubMed: 12356723]
- Zhou D, Chen LM, Hernandez L, Shears SB, Galan JE. A *Salmonella* inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization. Mol Microbiol 2001;39:248-259. [PubMed: 11136447]
- Boland A, Cornelis GR. Role of YopP in suppression of tumor necrosis factor alpha release by macrophages during *Yersinia* infection. Infect Immun 1998;66:1878-1884. [PubMed: 9573064]
- Schesser K, Spiik AK, Dukuzumuremyi JM, Neurath MF, Pettersson S, Wolf-Watz H. The yopJ locus is required for *Yersinia*-mediated inhibition of NF-kappaB activation and cytokine expression: YopJ

- contains a eukaryotic SH2-like domain that is essential for its repressive activity. *Mol Microbiol* 1998;28:1067–1079. [PubMed: 9680199]
9. Mota LJ, Cornelis GR. The bacterial injection kit: type III secretion systems. *Ann Med* 2005;37:234–249. [PubMed: 16019722]
 10. Fields KA, Mead DJ, Dooley CA, Hackstadt T. *Chlamydia trachomatis* type III secretion: evidence for a functional apparatus during early-cycle development. *Mol Microbiol* 2003;48:671–683. [PubMed: 12694613]
 11. Stephens RS, Kalman S, Lammel C, Fan J, Marathe R, Aravind L, Mitchell W, Olinger L, Tatusov RL, Zhao Q, et al. Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. *Science* 1998;282:754–759. [PubMed: 9784136]
 12. Slepentin A, Motin V, de la Maza LM, Peterson EM. Temporal expression of type III secretion genes of *Chlamydia pneumoniae*. *Infect Immun* 2003;71:2555–2562. [PubMed: 12704128]
 13. Matsumoto, A. Microbiology of Chlamydia. Barron AL: CRC Press; 1988. Structural characteristics of chlamydial bodies; p. 21-45.
 14. Bavoil PM, Hsia RC. Type III secretion in *Chlamydia*: a case of déjà vu? *Mol Microbiol* 1998;28:860–862. [PubMed: 9643553]
 15. Clifton DR, Fields KA, Grieshaber SS, Dooley CA, Fischer ER, Mead DJ, Carabeo RA, Hackstadt T. 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* 2004;101:10166–10171. [PubMed: 15199184]
 16. Jewett TJ, Fischer ER, Mead DJ, Hackstadt T. Chlamydial TARP is a bacterial nucleator of actin. *Proc Natl Acad Sci U S A* 2006;103:15599–15604. [PubMed: 17028176]
 17. Fields KA, Hackstadt T. Evidence for the secretion of *Chlamydia trachomatis* CopN by a type III secretion mechanism. *Mol Microbiol* 2000;38:1048–1060. [PubMed: 11123678]
 18. Slepentin A, de la Maza LM, Peterson EM. Interaction between components of the type III secretion system of Chlamydiaceae. *J Bacteriol* 2005;187:473–479. [PubMed: 15629918]
 19. Fields KA, Fischer ER, Mead DJ, Hackstadt T. Analysis of putative *Chlamydia trachomatis* chaperones Scc2 and Scc3 and their use in the identification of type III secretion substrates. *J Bacteriol* 2005;187:6466–6478. [PubMed: 16159780]
 20. Ouellette SP, Abdelrahman YM, Belland RJ, Byrne GI. The *Chlamydia pneumoniae* type III secretion-related lcrH gene clusters are developmentally expressed operons. *J Bacteriol* 2005;187:7853–7856. [PubMed: 16267309]
 21. Subtil A, Delevoye C, Balana ME, Tastevin L, Perrinet S, Dautry-Varsat A. A directed screen for chlamydial proteins secreted by a type III mechanism identifies a translocated protein and numerous other new candidates. *Mol Microbiol* 2005;56:1636–1647. [PubMed: 15916612]
 22. Subtil A, Parsot C, Dautry-Varsat A. Secretion of predicted Inc proteins of *Chlamydia pneumoniae* by a heterologous type III machinery. *Mol Microbiol* 2001;39:792–800. [PubMed: 11169118]
 23. Lugert R, Kuhns M, Polch T, Gross U. Expression and localization of type III secretion-related proteins of *Chlamydia pneumoniae*. *Med Microbiol Immunol* 2004;193:163–171. [PubMed: 14593477]
 24. Clifton DR, Dooley CA, Grieshaber SS, Carabeo RA, Fields KA, Hackstadt T. Tyrosine phosphorylation of the chlamydial effector protein Tarp is species specific and not required for recruitment of actin. *Infect Immun* 2005;73:3860–3868. [PubMed: 15972471]
 25. Forsberg A, Wolf-Watz H. The virulence protein Yop5 of *Yersinia pseudotuberculosis* is regulated at transcriptional level by plasmid-plB1-encoded trans-acting elements controlled by temperature and calcium. *Mol Microbiol* 1988;2:121–133. [PubMed: 2835586]
 26. Rivera-Amill V, Kim BJ, Seshu J, Konkel ME. Secretion of the virulence-associated Campylobacter invasion antigens from *Campylobacter jejuni* requires a stimulatory signal. *J Infect Dis* 2001;183:1607–1616. [PubMed: 11343209]
 27. Lee VT, Mazmanian SK, Schneewind O. A program of *Yersinia enterocolitica* type III secretion reactions is activated by specific signals. *J Bacteriol* 2001;183:4970–4978. [PubMed: 11489848]
 28. Bahrani FK, Sansonetti PJ, Parsot C. Secretion of Ipa proteins by *Shigella flexneri*: inducer molecules and kinetics of activation. *Infect Immun* 1997;65:4005–4010. [PubMed: 9316999]

29. van der Goot FG, Tran van Nhieu G, Allaoui A, Sansonetti P, Lafont F. Rafts can trigger contact-mediated secretion of bacterial effectors via a lipid-based mechanism. *J Biol Chem* 2004;279:47792–47798. [PubMed: 15364928]
30. Cornelis GR, Biot T, Lambert de Rouvroit C, Michiels T, Mulder B, Sluiter C, Sory MP, Van Bouchaute M, Vanootehem JC. The *Yersinia* yop regulon. *Mol Microbiol* 1989;3:1455–1459. [PubMed: 2693899]
31. Kim J, Ahn K, Min S, Jia J, Ha U, Wu D, Jin S. Factors triggering type III secretion in *Pseudomonas aeruginosa*. *Microbiology* 2005;151:3575–3587. [PubMed: 16272380]
32. Yahr TL, Hovey AK, Kulich SM, Frank DW. Transcriptional analysis of the *Pseudomonas aeruginosa* exoenzyme S structural gene. *J Bacteriol* 1995;177:1169–1178. [PubMed: 7868588]
33. Menard R, Sansonetti P, Parsot C. The secretion of the *Shigella flexneri* Ipa invasins is activated by epithelial cells and controlled by IpaB and IpaD. *Embo J* 1994;13:5293–5302. [PubMed: 7957095]
34. Menard R, Sansonetti PJ, Parsot C. Nonpolar mutagenesis of the ipa genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. *J Bacteriol* 1993;175:5899–5906. [PubMed: 8376337]
35. Watarai M, Tobe T, Yoshikawa M, Sasakawa C. Contact of *Shigella* with host cells triggers release of Ipa invasins and is an essential function of invasiveness. *EMBO J* 1995;14:2461–2470. [PubMed: 7781600]
36. Stuart ES, Webley WC, Norkin LC. Lipid rafts, caveolae, caveolin-1, and entry by Chlamydiae into host cells. *Exp Cell Res* 2003;287:67–78. [PubMed: 12799183]
37. Gabel BR, Elwell C, van Ijzendoorn SC, Engel JN. Lipid raft-mediated entry is not required for *Chlamydia trachomatis* infection of cultured epithelial cells. *Infect Immun* 2004;72:7367–7373. [PubMed: 15557670]
38. Tipples G, McClarty G. The obligate intracellular bacterium *Chlamydia trachomatis* is auxotrophic for three of the four ribonucleoside triphosphates. *Mol Microbiol* 1993;8:1105–1114. [PubMed: 8361355]
39. Muschiol S, Bailey L, Gylfe A, Sundin C, Hultenby K, Bergstrom S, Elofsson M, Wolf-Watz H, Normark S, Henriques-Normark B. A small-molecule inhibitor of type III secretion inhibits different stages of the infectious cycle of *Chlamydia trachomatis*. *Proc Natl Acad Sci U S A* 2006;103:14566–14571. [PubMed: 16973741]
40. Wolf K, Betts HJ, Chellas-Gery B, Hower S, Linton CN, Fields KA. Treatment of *Chlamydia trachomatis* with a small molecule inhibitor of the Yersinia type III secretion system disrupts progression of the chlamydial developmental cycle. *Mol Microbiol* 2006;61:1543–1555. [PubMed: 16968227]
41. Caldwell HD, Kromhout J, Schachter J. Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis*. *Infect Immun* 1981;31:1161–1176. [PubMed: 7228399]
42. Furness G, Graham DM, Reeve P. The titration of trachoma and inclusion blennorrhoea viruses in cell cultures. *J Gen Microbiol* 1960;23:613–619. [PubMed: 13702673]
43. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685. [PubMed: 5432063]
44. Schroeder RJ, Ahmed SN, Zhu Y, London E, Brown DA. Cholesterol and sphingolipid enhance the Triton X-100 insolubility of glycosylphosphatidylinositol-anchored proteins by promoting the formation of detergent-insoluble ordered membrane domains. *J Biol Chem* 1998;273:1150–1157. [PubMed: 9422781]
45. Carabeo RA, Grieshaber SS, Fischer E, Hackstadt T. *Chlamydia trachomatis* induces remodeling of the actin cytoskeleton during attachment and entry into HeLa cells. *Infect Immun* 2002;70:3793–3803. [PubMed: 12065523]
46. Baehr W, Zhang YX, Joseph T, Su H, Nano FE, Everett KD, Caldwell HD. Mapping antigenic domains expressed by *Chlamydia trachomatis* major outer membrane protein genes. *Proc Natl Acad Sci U S A* 1988;85:4000–4004. [PubMed: 2453883]

FIG. 1.

Stability of EBs in various buffers. *C. trachomatis* L2 EBs were incubated in buffers at 37°C for 30 minutes. The supernatant was collected and analyzed by silver staining and immunoblotting. Buffers tested were: 50 mM KAcetate buffer, pH 4.8 (KAc), SPG buffer (SPG), Hank's balanced salt solution (HBSS), PBS (PBS), .25 M sucrose (Sucrose), and water. The supernatants were electrophoresed, then silver-stained to visualize protein. In addition, the supernatants were analyzed by immunoblotting with an α-Tarp antibody (α-Tarp) and an α-EB antibody (α-EB). Approximately 5×10^7 EBs were loaded on the gels as a control.

FIG. 2.

FBS, BSA, and SCRLs induce T3S from chlamydial EBs. The induction conditions tested were: 50 mM KAcetate buffer, pH 4.8 (KAc), 3% fetal bovine serum in 50 mM KAc, pH 4.8 (FBS), 0.5% bovine serum albumin in 50 mM KAc, pH 4.8 (BSA), 135 μ M glutamate in 50 mM KAc, pH 4.8 (Glutamate), 10 μ M congo red in 50 mM KAc, pH 4.8 (Congo Red), and SCRLs in 50 mM KAc, pH 4.8 (liposomes). The supernatant was immunoblotted with an α -TarP antibody (α -TarP) and then stripped and re-probed with an anti-EB antibody (α -EB). Arrowhead indicates the position of TarP. Several apparent TarP breakdown products were detected in the EBs. Note that the volume of EBs loaded in the EB lane is equivalent to only 0.3% of the quantities induced to generate the supernatants loaded in the other lanes. The figure was prepared from single exposures of immunoblots against anti-TarP and anti-EB antisera, respectively. The liposome and EB lanes were spliced next to the remaining lanes for presentation purposes using Adobe Photoshop without other manipulation.

FIG. 3.

EGTA enhances the amount of TarP secreted during induction with BSA and FBS. One tenth volume of 100 mM EGTA (or water) was added to the induction solutions. The conditions were: 50 mM KAcetate buffer (KAc), 50 mM KAc buffer with water (KAc + water), 50 mM KAc buffer with 10 mM EGTA (KAc + EGTA), 3% FBS with water (FBS + water), 3% FBS with 10 mM EGTA, 0.5% BSA with water (BSA + water), and 0.5% BSA with 10 mM EGTA (BSA + EGTA). The supernatants were collected and analyzed by immunoblotting with an α -TarP antibody (α -TarP). EBs were loaded as a control for the antibody. The lane loaded with EBs was exposed for for a shorter interval due to a very strong signal.

FIG. 4.

T3S in chlamydia is temperature sensitive. Using SCRLs (Liposomes) as the positive control for induction and 50 mM KAcetate buffer, pH 4.8 (KAc), as the negative control, EBs were induced at either 4°C or 37°C for 30 minutes. The supernatants were collected and analyzed by immunoblotting with an α -Tarp antibody.

FIG. 5.

A majority of Tarp remains associated with EBs after induction. EBs were induced with 50 mM KAcetate, pH 4.8 (KAc) as a negative control, or with SCRLs in 50 mM KAcetate, pH 4.8 (Liposomes) or 0.5% BSA in 50 mM KAcetate, pH 4.8 (BSA). EBs or EBs after BSA induction were collected for analysis of Tarp by SDS-PAGE and immunoblotting with an anti-Tarp antibody. Note that due to the large amounts of Tarp remaining in EBs after induction, the volume of EBs loaded is equivalent to only 0.3% of the quantities induced to generate the supernatants loaded in the KAc, Liposomes, and BSA lanes.

FIG. 6.

Paraformaldehyde fixation of *C. trachomatis* EBs prevents induction of the T3S. (A) EBs were fixed in 4% paraformaldehyde and then induced with SCRLs following the protocol previously described. The supernatants were collected and analyzed by immunoblotting with an α -Tarp antibody (α -Tarp). (B) Paraformaldehyde fixation of EBs prevents translocation of the Tarp protein, as evidenced by lack of tyrosine phosphorylation, and entry of EBs into HeLa cells. Shown here are unfixed, control EBs (Con), 2.5% glutaraldehyde-fixed EBs (Glut), 4% paraformaldehyde-fixed EBs (pHCHO), and uninfected cells (Unif). After 30 min incubation at 37°, the cells were fixed and immunostained using a rabbit α -EB antibody (α EB) and a mouse monoclonal α -phosphotyrosine antibody (α pY).

Table 1

In vitro induction of T3S does not cause loss of EB infectivity.

Buffer	Mean $\pm$ S.D.
SPG	$9.97 \times 10^9 \pm 5.42 \times 10^8$
KAc	$3.69 \times 10^9 \pm 1.81 \times 10^8$
SCRL	$7.51 \times 10^9 \pm 4.2 \times 10^8$
PBS	$6.92 \times 10^9 \pm 2.75 \times 10^8$
BSA	$3.68 \times 10^9 \pm 2.96 \times 10^8$