

Altered protein secretion of *Chlamydia trachomatis* in persistently infected human endocervical epithelial cells

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Chlamydia trachomatis is the most common bacterial infection of the human reproductive tract globally; however, the mechanisms underlying the adaptation of the organism to its natural target cells, human endocervical epithelial cells, are not clearly understood. To secure its intracellular niche, *C. trachomatis* must modulate the host cellular machinery by secreting virulence factors into the host cytosol to facilitate bacterial growth and survival. Here we used primary human endocervical epithelial cells and HeLa cells infected with *C. trachomatis* to examine the secretion of bacterial proteins during productive growth and persistent growth induced by ampicillin. Specifically, we observed a decrease in secretable chlamydial protease-like activity factor (CPAF) in the cytosol of host epithelial cells exposed to ampicillin with no evident reduction of CPAF product by *C. trachomatis*. In contrast, the expression of CopN and Tarp was downregulated, suggesting that *C. trachomatis* responds to ampicillin exposure by selectively altering the expression of secretable proteins. In addition, we observed a greater accumulation of outer-membrane vesicles from *C. trachomatis* in persistently infected cells. Taken together, these results suggest that the regulation of both gene expression and the secretion of chlamydial virulence proteins is involved in the adaptation of the bacteria to a persistent infection state in human genital epithelial cells.

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INTRODUCTION

Genital serovariants (serovars D–K) of the obligate intracellular bacterium *Chlamydia trachomatis* are the world's most common sexually transmitted bacterial pathogens, accounting for an estimated 90 million new cases annually (Brunham & Rey-Ladino, 2005). These

serovariants have an exclusive tropism for the columnar epithelial cells of the genital mucosae. A typical infection cycle is initiated when infectious elementary bodies (EBs) attach to and enter into these cells. Subsequently, bacteria reside within a membrane-bound vacuole termed an inclusion and undergo a programmed biphasic transition from EBs to metabolically active replicating reticulate

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Abbreviations: CPAF, chlamydial protease-like activity factor; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; DIC, differential interference contrast; EB, elementary body; IFA, immunofluorescence analysis; IFN- γ , interferon-gamma; IFU, inclusion-forming unit; MHC, major histocompatibility complex; MOMP, major outer-membrane protein; OMV, outer-membrane vesicle; p.i., post-infection; RB, reticulate body; TEM, transmission electron microscopy; TTSS, type III secretion system.

bodies (RBs), and then from RBs to EBs (Hatch, 1999; Moulder, 1991). Eventually, EBs and RBs exit the cell by extrusion or cell lysis (Hybiske & Stephens, 2007). *Chlamydia* species can also enter into an altered growth state that has been termed persistence. Chlamydial persistence *in vitro* is defined as a viable but non-cultivable growth state and is typified by enlarged, pleiomorphic RBs that cannot undergo binary fission and differentiation into EBs; however, they do continue chromosomal and plasmid replication (Beatty *et al.*, 1994; Hogan *et al.*, 2004; Lambden *et al.*, 2006). Although antibiotics usually resolve infection, these aberrant persistent forms are more refractory to antibiotic treatment in culture (Reveneau *et al.*, 2005; Wyrick & Knight, 2004). Several inducers have been shown to cause persistent forms *in vitro* (Wyrick, 2010), including exposure to penicillin or ampicillin (Matsumoto & Manire, 1970; Wolf *et al.*, 2000), exposure to interferon-gamma (IFN- γ) (Beatty *et al.*, 1994; Belland *et al.*, 2003), nutrient depletion (Harper *et al.*, 2000; Raulston, 1997) and co-infection of epithelial cells with herpes simplex virus (Deka *et al.*, 2006).

The most common genital site of infection in women is the endocervix. Infection may result in the clinical syndrome of mucopurulent cervicitis but it is asymptomatic in over 80% of individuals (Brunham & Rey-Ladino, 2005). Natural history studies suggest that most women can eventually clear the infection, but this can take up to several years (Brunham & Rey-Ladino, 2005; Gottlieb *et al.*, 2010; Molano *et al.*, 2005). In some women, *C. trachomatis* may ascend into the endometrium and fallopian tubes, where the bacteria can establish a chronic infection that results in pelvic inflammatory disease. Why *Chlamydia* infections take so long to clear is not known, but it is thought to be the consequence of the numerous evasion strategies used by the organism, including the ability to persist as an altered intracellular form (Brunham & Rey-Ladino, 2005).

To secure an intracellular niche, *Chlamydia* must secrete virulence proteins into the host cell, where they modulate the host cellular machinery to promote infection (Betts *et al.*, 2009). *Chlamydia* species utilize not only the type III secretion system (T3SS), but also other diverse secretion mechanisms to translocate bacterial proteins into host cells. A major virulence factor, chlamydial protease-like activity factor (CPAF), has been shown to translocate via the Sec-dependent pathway (Chen *et al.*, 2010). CPAF primarily interferes with cellular processes by cleaving host proteins, including the transcription factors RFX and USF-1 that are required for major histocompatibility complex (MHC) antigen expression (Zhong *et al.*, 2001). Some T3SS effectors are also known to target various cellular processes. For example, secretion of the T3SS effectors Tarp (Clifton *et al.*, 2004) and CT694 (Hower *et al.*, 2009) from EBs into the host cytosol facilitates *C. trachomatis* entry via actin recruitment. Additionally, CopN serves as a virulence factor (Huang *et al.*, 2008) and as an exported T3SS regulator that modulates the secretion of T3SS translocators

and effectors (Fields & Hackstadt, 2000). Members of the family of inclusion-membrane proteins (Inc), such as IncA, directly participate in inclusion biogenesis, acquisition of host nutrients and interaction with the host protein factors (Bannantine *et al.*, 1998; Rockey *et al.*, 2002). Elucidation of the machinery and the conditions that trigger secretion of virulence proteins from *C. trachomatis* into the cytosol of host cells would provide new insights for understanding the pathogenesis of the disease.

Previous studies have indicated that *in vitro* inducers of persistence, including iron depletion or IFN- γ treatment of infected HEp-2 cells, decrease the secretion of CPAF protein by *C. pneumoniae* (Heuer *et al.*, 2003; Shaw *et al.*, 2002). However, relationships between secretion activities, gene regulation, and the mechanism by which *C. trachomatis* survives under conditions that induce persistence, remain to be determined. Here, using primary epithelial cells derived from the human endocervix, the most common natural tissue site of *C. trachomatis*, and the classic HeLa cell infection model, we determined whether the secretion activity of *C. trachomatis* changed in the presence of ampicillin and explored some of the possible mechanisms underlying these changes. As a commonly prescribed antibiotic for treatment of bacterial infections, ampicillin has been used as an inducer of persistence *in vitro* and it simulates an inadequate antimicrobial treatment of *Chlamydia* infection (Wyrick, 2010). We demonstrated that ampicillin exposure of infected epithelial cells interrupted the development of *C. trachomatis* and redistributed CPAF. We also confirmed our observations in the IFN- γ -mediated model of *C. trachomatis* persistence (Belland *et al.*, 2003). These changes in the bacteria–host cell interaction during persistence could provide an advantage to *C. trachomatis* for survival and adaptation in human endocervical epithelial cells.

METHODS

Cell culture and *C. trachomatis* infection. HeLa 229 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and 10 μg gentamicin ml^{-1} . Human primary endocervical epithelial cell cultures were established from endocervical tissue explants obtained from women undergoing hysterectomies for benign gynaecological conditions under a protocol approved by the LSU Health Sciences Center Institutional Review Board as previously described (Herbst-Kralovetz *et al.*, 2008). Primary endocervical epithelial cells were maintained at 37 °C in 5% CO₂ in a serum-free medium that is highly selective for epithelial cells: EpiLife medium containing EpiLife defined growth supplement (Cascade Biologics). Monolayers of HeLa 229 cells or primary endocervical cells were inoculated with *C. trachomatis* serovar F/Cal-I-13 with a dose that results in 50% of cells being infected, and centrifuged at 1600 g for 40 min at 37 °C. Fresh medium was added to the infected cells and incubated at 37 °C for various time periods as indicated in each experimental result.

Ampicillin and IFN- γ exposure of *C. trachomatis*-infected cells and persistence reactivation assays. Infected HeLa and primary human endocervical epithelial cells were exposed to ampicillin (10 μg ml^{-1}) at 16 h post-infection (p.i.). A reactivation experiment was performed in both HeLa and primary endocervical cells by removing

ampicillin-containing medium from the culture after 8 h of ampicillin exposure (at 24 h p.i.), followed by incubation with ampicillin-free medium for an additional 12 or 32 h (Fig. 1). Subculture was performed to enumerate infectious progeny (i.e. EBs) through the calculation of inclusion-forming units (IFUs).

The IFN- γ model of chlamydial persistence was performed as described by Belland *et al.* (2003), but with minor modifications. Briefly, HeLa and primary human endocervical epithelial cells were exposed to 50 or 200 U IFN- γ ml⁻¹, respectively, for 24 h prior to infection. After infection, fresh IFN- γ -containing medium was added and maintained until fixation at 36 h p.i. A concentration of 50 U IFN- γ ml⁻¹ was used for infected HeLa cells, and 200 U IFN- γ ml⁻¹ was used for infected primary endocervical cells since there is a high concentration of tryptophan in the primary culture medium. The optimal IFN- γ concentrations that resulted in a persistent phenotype were determined using morphological analyses and subculture techniques to enumerate infectious progeny. The ability to reactivate persistent bacteria was confirmed using the standard methodology of Belland *et al.* (2003).

Purification of *C. trachomatis* particles from infected HeLa cells. RBs and EBs were purified by centrifugation on Renografin density gradients as previously described (Shen *et al.*, 2000). To isolate fragile persistent forms (PBs), cell disruption by nitrogen cavitation was used, which is a technique based on the rapid decompression of a cell suspension from a pressure vessel (Gottlieb & Adachi, 2000). For 2.5×10^8 infected HeLa cells, we used 125 p.s.i. (862 kPa) for 5 min intervals until more than 90% host cell lysis was achieved, as determined by trypan blue staining. A solution of 25% Renografin in sucrose/phosphate/glutamic acid buffer (SPG: 0.2 M sucrose, 3.8 mM KH₂PO₄, 6.7 mM Na₂HPO₄, 5 mM L-glutamic acid, pH 7.4) was overlaid with a 5 ml sample of infected cells and centrifuged in a Beckman SW 28 rotor at 18000 r.p.m. for 1 h at 4 °C. The resuspended bacterial mixture was then overlaid on and centrifuged through a discontinuous gradient consisting of 28%, 32%, 40%, 44% and 52% Renografin in a SW 28 rotor at 24000 r.p.m. for 1 h at 4 °C. The bands of turbidity at interfaces of 28–34% and 34–40% Renografin solution were collected, sedimented by centrifugation, and suspended in 2 ml SPG for analysis by transmission electron microscopy (TEM) and immunoblotting.

Antibodies. The primary antibodies used to detect chlamydial antigens in this study were (i) rabbit polyclonal antibodies directed against CopN (kind gift of Dr Ken Fields, University of Miami, FL, USA), IncA and Tarp (kind gifts of Dr Ted Hackstadt, Rocky Mountain Laboratories, NIH, NIAID, Hamilton, MT, USA) and OmcB (a kind gift of Dr Patrick Bavoil, University of Maryland, MD, USA); (ii) mouse monoclonal antibodies against the major outer-membrane protein (MOMP) (Wang *et al.*, 2006) and EF-Tu (kind gifts of Dr You-xun Zhang, Boston University, Boston, MA, USA), and the C-terminal region of CPAF (clone 100) (Zhong *et al.*, 2001).

Indirect immunofluorescence assays. *C. trachomatis*-infected HeLa and primary endocervical cells on 12 mm-diameter glass coverslips were fixed with 2% paraformaldehyde for 45 min and

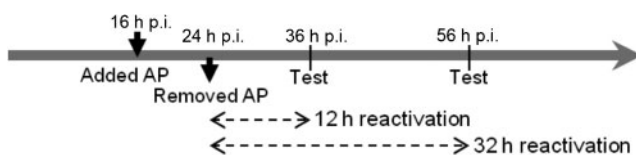


Fig. 1. Experimental schematic for the reactivation assay.

permeabilized with 4% saponin for 30 min, followed by blocking with iTFX (Invitrogen) overnight at 4 °C. Primary antibodies against chlamydial MOMP, IncA, CPAF, CopN or Tarp were incubated with cells overnight at 4 °C, followed by either Alexa 568- or FITC-conjugated secondary antibodies (Molecular Probes) for 45 min at 37 °C. All cells were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes). Images were obtained with a Nikon E600 and Leica DMRXA automated upright epifluorescent microscope. Z-axis plane capture, deconvolution and analyses were performed with SlidebookTM deconvolution software (Intelligent Imaging Innovations).

Immunoprecipitation and immunoblot analysis. A Subcellular Protein Fractionation kit (Thermo Fisher Scientific) was used to separate and extract different cellular contents stepwise from *C. trachomatis*-infected cells at 4 °C, according to the manufacturer's instructions. Briefly, cytoplasmic extraction buffer was added to the cell pellet to cause selective membrane permeabilization, releasing the soluble host cell cytoplasmic fraction. Membrane extraction buffer was used to solubilize plasma, mitochondrial and endoplasmic reticulum–Golgi membranes. Nuclear extraction buffer was used to solubilize nuclear and chlamydial inclusion membranes and inclusion contents. The remaining pellet contained mostly actin-bound protein and insoluble chlamydial proteins. For simplification of nomenclature in this study, these fractions were designated cytosol, membrane, inclusion fraction and pellet, respectively. For immunoprecipitation, soluble cellular fractions dialysed against buffer containing 10 mM Tris/HCl (pH 8.0), 200 mM NaCl, 1 μ M DTT and 5% (v/v) glycerol were mixed with different specific antibodies and incubated at 4 °C overnight. Protein and antibody complexes were captured with protein A Sepharose (Sigma) for an additional 6 h at 4 °C. After extensive washing to remove non-specifically bound material, protein complexes were then separated by 12% SDS-PAGE, transferred to Immobilon-PVDF membranes and subjected to immunoblot analysis as described previously (Hua *et al.*, 2009). Blots were developed using a horseradish peroxidase-conjugated secondary antibody and the SuperSignalP Chemiluminescent Detection kit (Pierce). Density of the signals on the immunoblot was determined using Quantity One software (Bio-Rad) (Shen *et al.*, 2004).

Total RNA extraction and RT-PCR analysis. Total RNA was isolated from cells harvested at 24, 36, 40 and 48 h p.i. using TRIzol (Invitrogen), according to the manufacturer's instructions. Samples were treated with DNase I (USB) to remove contaminating DNA, as described previously (Rao *et al.*, 2009). Primer pairs were designed to amplify products of 332 bp for the *tuf* gene (CT322), 200 bp for the *cpaf* gene (CT858), 213 bp for the *copN* gene (CT089), and 164 bp for the *tarp* gene (CT456). They are *tuf*FrF and *tuf*FrR (5'-GACAAGTTG-GGGTTCCTTAC-3' and 5'-ACGCTCAATACGTCCAGTGA-3'), *cpaf*FrF and *cpaf*FrR (5'-CTCAGGATGAAGTGGTTGAT-3' and 5'-GAATAG-GCGTTGATAACTCG-3'), *copN*FrF and *copN*FrR (5'-AACTGGTAG-GCCCAGATACT-3' and 5'-GAGGCGTACTAGAGAAGGAAG-3') and *tarp*FrR3 and *tarp*FrR3 (5'-AGTTACACAACAGCAAACG-3' and 5'-ACACTTCTGAATCATCTCC-3). Five micrograms of total RNA was reverse transcribed in a reaction containing reverse primers and 5 U AMV reverse transcriptase (USB) at 42 °C for 1 h. PCR was performed on the cDNA products, with the primer pairs as above, and *Taq* DNA polymerase (New England Biolabs) using the following protocol: 94 °C 0.5 min, 54 °C 0.5 min, and 72 °C 0.5 min for 30 cycles. PCR products were separated on a 2.0% agarose gel, stained with ethidium bromide, photographed with a Gel Doc 3000 (Bio-Rad), and then quantified using Quantity One software (Bio-Rad) as described previously (Shen *et al.*, 2004).

Electron microscopy. For ultrastructural analysis, infected HeLa and primary endocervical epithelial cells or Renografin-purified *C.*

trachomatis forms were fixed and processed as described previously (Beatty, 2006).

RESULTS

Establishment of *C. trachomatis* infection in primary human endocervical epithelial cells

Previous studies have indicated that cultured primary human endometrial and ectocervical epithelial cells are less susceptible to *C. trachomatis* infection compared to McCoy cells (Moorman *et al.*, 1986). To establish whether normal *C. trachomatis* forms could develop in primary human

endocervical epithelial cells, we used 10 times more EBs than the number needed for a 50% infection in HeLa cells to achieve equivalent infection rates in both cell types. Depending on the donor ($n=7$), 30–70% of cells were infected as determined by immunofluorescence analysis (IFA) using an anti-MOMP antibody. *C. trachomatis* inclusions in primary endocervical cell cultures exhibited an irregular staining pattern of MOMP compared to that observed in HeLa cells (Fig. 2A, B). The size and morphology of inclusions in these primary cell cultures varied from donor to donor, but no correlation was found between the size of the inclusion and the size of the cell when the inclusion area was normalized to the area of the cell (data not shown). TEM analysis revealed that

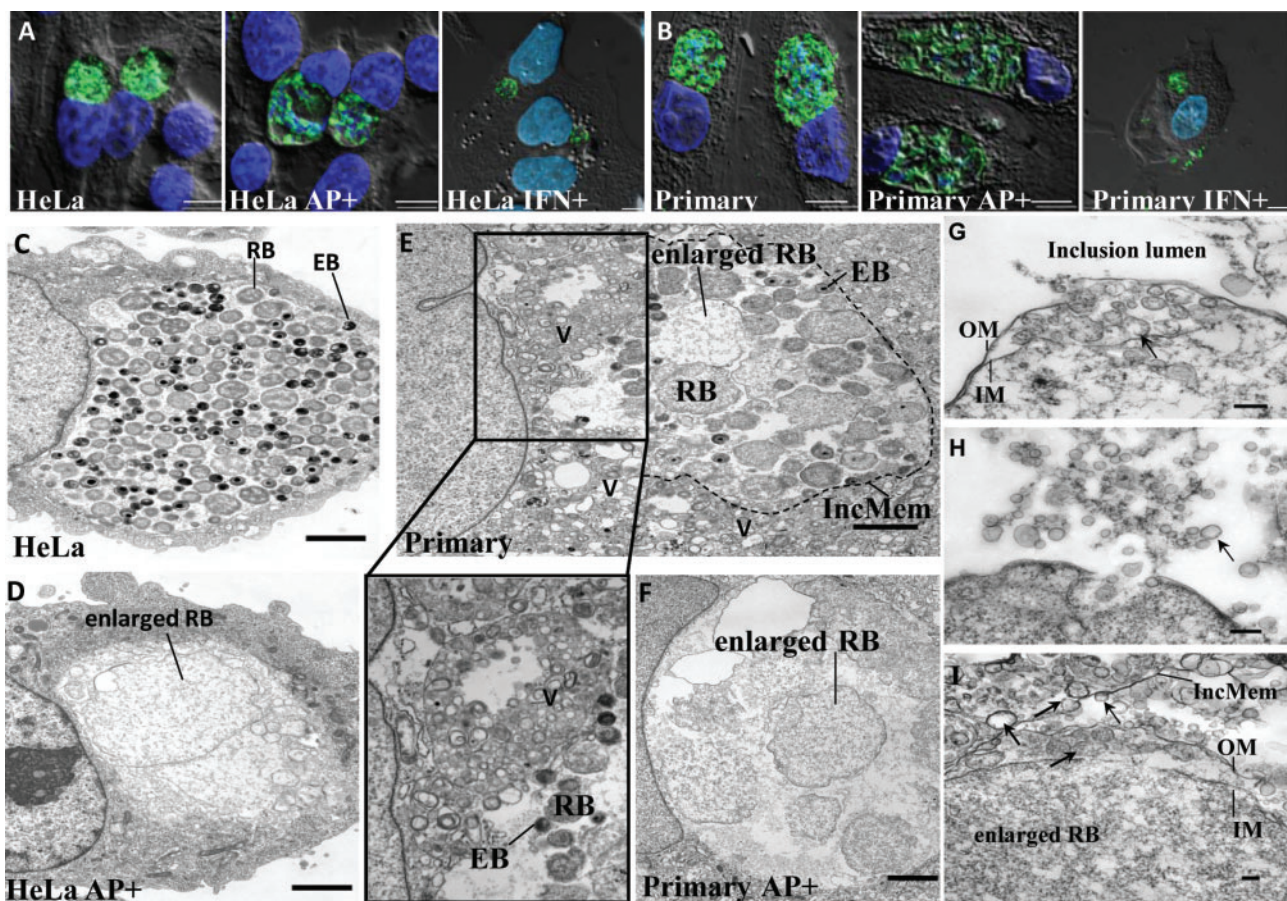


Fig. 2. *C. trachomatis* infection in genital tract epithelial cells. (A, B) Morphological analysis by epifluorescent microscopy and deconvolution of *C. trachomatis*-infected cells unexposed or exposed to ampicillin (AP+) or IFN- γ (IFN+). Infected HeLa cells (A) and primary human endocervical epithelial cells (B) were fixed at 36 h p.i. MOMP was visualized as green. Host nuclei and bacteria were visualized as blue by DAPI staining. All images shown are overlays of fluorescence images (green and blue) on differential interference contrast (DIC) images (grayscale). Scale bars, 5 μ m. (C, D) TEM of *C. trachomatis* inclusions reveals the presence of productive forms in the absence of ampicillin (C) and the accumulation of enlarged persistent forms in the presence of ampicillin (D) in *C. trachomatis*-infected HeLa cells. Scale bars, 2 μ m. (E, F) TEM of *C. trachomatis* inclusions in infected primary human endocervical epithelial cells unexposed (E) or exposed to ampicillin (F). An enlarged image of multivesicular structures from (E) is shown below. V, multivesicular structures; IncMem, inclusion membrane (outlined with dotted line). Scale bars, 2 μ m. (G–I) *C. trachomatis* produces OMVs in cultured primary human endocervical epithelial cells exposed to ampicillin as determined by TEM. OM/IM, outer/inner membrane; IncMem, inclusion membrane. Representative OMVs are indicated by arrows. Scale bars, 200 nm (G, H) or 100 nm (I).

inclusions generally contained fewer chlamydial particles in primary endocervical cells (Fig. 2E) than those seen in HeLa cells (Fig. 2C). In infected primary endocervical cells fixed at 36 h p.i., there was a mixture of normal EBs, RBs and intermediate forms, features suggestive of productive chlamydial forms. Additionally, enlarged RB-like forms with lower electron density often co-existed in *C. trachomatis*-infected primary endocervical cells. We also observed that some of the membranes of the enlarged forms as well as the inclusions were disrupted in the cross-section view. These data indicate that primary human endocervical epithelial cells are susceptible to *C. trachomatis* infection *ex vivo*. Interestingly, there were abundant multivesicular structures in the cytosol of infected primary endocervical epithelial cells that were adjacent to the inclusion (Fig. 2E). These multivesicular structures were also observed in *C. trachomatis*-infected HeLa cells, but in much lower numbers (data not shown). In the cross-section view of cells these multivesicular structures were generally round, with diameters ranging from 100 to 300 nm. No similar structures were observed in uninfected cells, suggesting that the formation of these multivesicular structures was associated with *C. trachomatis* infection.

Ampicillin and IFN- γ induce viable but non-cultivable persistent forms in HeLa and primary endocervical epithelial cells

Exposure of *C. trachomatis* serovar F-infected HeLa and primary endocervical cells to ampicillin or IFN- γ resulted in aberrant, enlarged pleiomorphic RBs as determined by IFA with anti-MOMP staining (Fig. 2A, B). TEM imaging was also utilized to visualize ampicillin-mediated persistent forms (Fig. 2C, D). These results are consistent with observations from other investigators (Matsumoto & Manire, 1970; Wolf *et al.*, 2000; Wyrick & Knight, 2004).

Infection assays were performed to evaluate infectious chlamydial forms in both *C. trachomatis*-infected HeLa and primary endocervical cells exposed to ampicillin. IFUs

recovered from exposed cultures were negligible, consistent with a viable but non-cultivable phenotype (Table 1). Reactivation from persistence was effective in recovering infectious forms after removal of ampicillin. The recovered IFUs from reactivation were similar in number to those found in unexposed cultures (Table 1). The reactivated inclusions contained normal RBs, EBs and intermediate bodies, similar to those observed in the normal infected cultures.

Together these data indicate that despite slight phenotypic differences, persistent *C. trachomatis* forms can be induced by ampicillin and IFN- γ in both primary endocervical and HeLa cells. Thus, the generation of abnormal persistent forms of *C. trachomatis* is not an artefact of chlamydial growth in transformed cell lines.

Ampicillin exposure increases the formation of OMVs in HeLa and primary endocervical epithelial cells

TEM analysis showed that ampicillin exposure induced an accumulation of small spherical bilayered vesicles in the size range of 30 to 200 nm as viewed in cross-section (Fig. 2G–I). The presence of these vesicles corresponded to the reported OMVs or blebs observed in other cell lines infected with *Chlamydia* spp. (Giles *et al.*, 2006; Matsumoto & Manire, 1970; Stirling & Richmond, 1980). The vesicles were observed between the inner and outer bacterial membranes, and this observation was highly reproducible in both *C. trachomatis*-infected primary endocervical cells and HeLa cells. TEM images suggest that OMVs may be released from the bacterial cells into the inclusion lumen (Fig. 2H, I). These vesicles appear to also be able to attach to the inclusion membrane (Fig. 2I) and can also be found in the host cell cytosol (Fig. 2I). In contrast, few OMVs were observed in *C. trachomatis*-infected HeLa and primary endocervical cells unexposed to ampicillin, and were more commonly associated with RBs rather than the inclusion membrane (data not shown).

Table 1. Reactivation assay and recovery of IFUs from infected cells

See Methods and Fig. 1 for details of the reactivation assay. IFU counts are means \pm SD from three replicates for each sample. AP, ampicillin exposure; RA, reactivation.

Sample	No. of IFUs recovered			
	DTCRC2*	DTCRC3*	DTCRC66*	HeLa cells
36 h	$(3.88 \pm 2.52) \times 10^2$	$(4.96 \pm 2.8) \times 10^5$	$(1.01 \pm 0.13) \times 10^5$	$(3.05 \pm 0.21) \times 10^6$
36 h AP	2.82 ± 0.58	3.10 ± 0.30	4.50 ± 0.20	8.95 ± 2.90
36 h RA	$(3.84 \pm 2.16) \times 10^2$	$(4.62 \pm 0.63) \times 10^5$	$(2.70 \pm 1.4) \times 10^5$	$(2.88 \pm 0.14) \times 10^6$
56 h	$(3.91 \pm 1.9) \times 10^2$	$(3.44 \pm 3.1) \times 10^5$	$(2.42 \pm 0.24) \times 10^5$	$(3.21 \pm 0.71) \times 10^6$
56 h AP	4.42 ± 1.6	3.15 ± 0.10	4.30 ± 0.10	6.60 ± 1.12
56 h RA	$(4.21 \pm 2.5) \times 10^2$	$(4.62 \pm 0.41) \times 10^5$	$(3.25 \pm 2.3) \times 10^5$	$(2.89 \pm 0.71) \times 10^6$

*Primary epithelial cells from three donors, DTCRC2, DTCRC3 and DTCRC66.

Generation of persistent forms of *C. trachomatis* is coupled with altered localization of secretable bacterial proteins in infected cells

As a step to determine the relationship between bacterial secretion and the persistent state, we assessed the cellular localization of secretable proteins, namely CPAF and two T3SS effectors, Tarp (Clifton *et al.*, 2004) and CopN (Fields & Hackstadt, 2000), during persistence. The T3SS effector IncA (Bannantine *et al.*, 1998; Rockey *et al.*, 2002) was used to visualize the inclusion membrane.

Time-course experiments of CPAF protein expression in normal *C. trachomatis* serovar F-infected HeLa cells indicated that CPAF-specific signals were present in the inclusion by 16 h p.i. By 24 h p.i., CPAF had clearly translocated into the host cell cytosol, where it remained for up to 48 h p.i. (36 h time point shown in Fig. 3A). This is consistent with previous observations using HeLa cells infected with *C. trachomatis* serovars A, D and L2 (Heuer *et al.*, 2003; Zhong *et al.*, 2001). Similarly, CPAF localized to the host cell cytosol in primary human endocervical epithelial cells at 36 h p.i. (Fig. 3D). Exposure to ampicillin

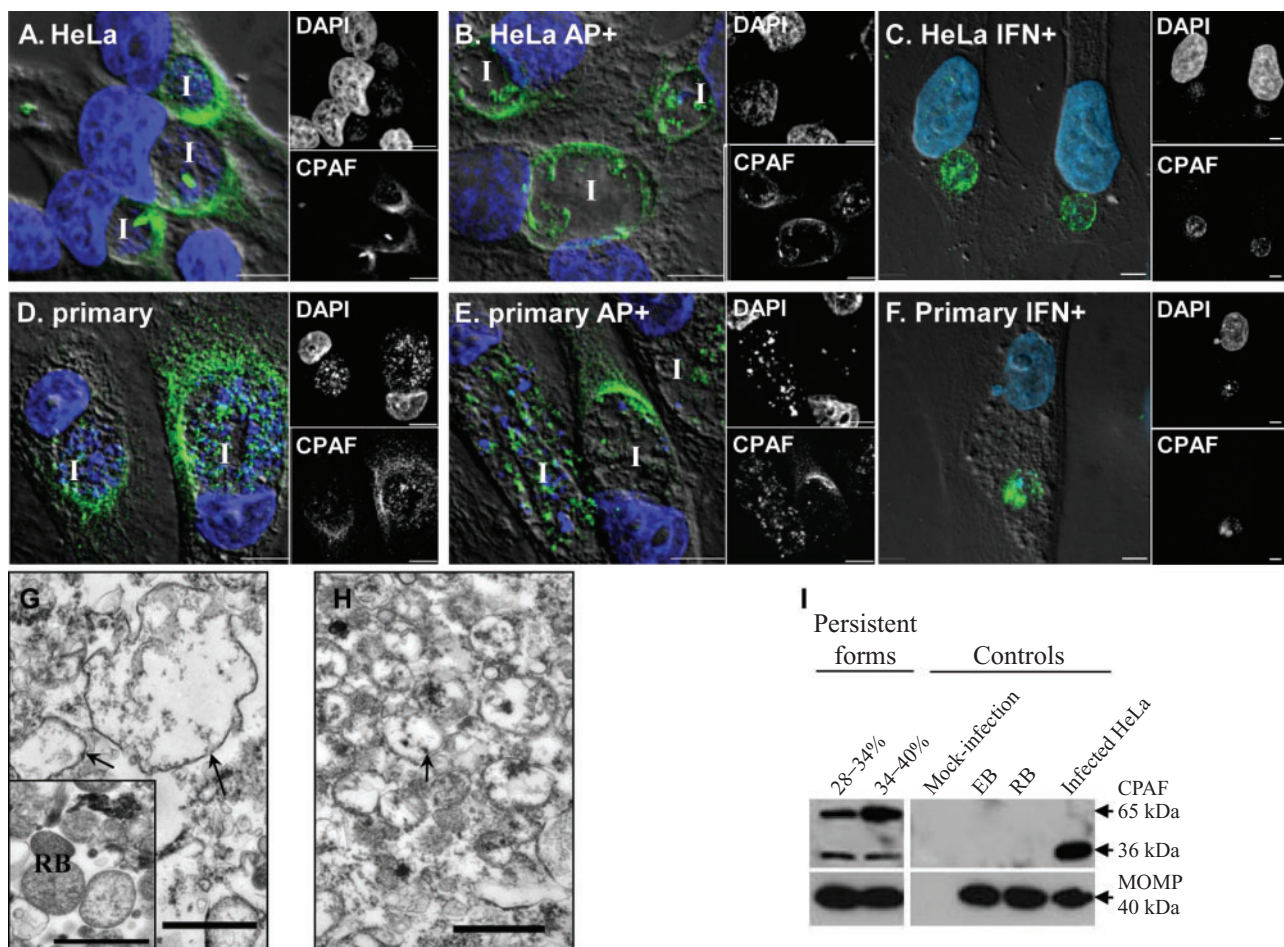


Fig. 3. Localization of CPAF protein in *C. trachomatis*-infected cells. Infected HeLa cells (A–C) and primary endocervical cells (D–F) cultured in the absence or presence of persistence inducers (ampicillin, AP +; or IFN- γ , IFN +) as indicated were fixed at 36 h p.i. and immunostained with anti-CPAF antibody 100a. Overlays of DIC, CPAF (green) and DAPI (blue) images are shown in the large panels to the left, with separate small images of the single channels to the right. I, inclusion. Scale bars, 5 μ m. (G, H) Representative TEM images of persistent forms isolated from *C. trachomatis*-infected HeLa cells exposed to ampicillin at the 28–34% Renografin interface (G) and the 34–40% Renografin interface (H). The control image of isolated normal RBs is shown in the inset in (G). Representative persistent forms are indicated by arrows. Note the difference in electron density between normal RBs and the persistent forms. Scale bars, 1 μ m. (I) Immunoblot analysis of isolated *C. trachomatis* forms against CPAF. Lysates of isolated persistent forms at the 28–34% and 34–40% interfaces (left panels), and control RBs and EBs (right panels), were fractionated by SDS-PAGE, followed by immunoblotting with anti-CPAF antibody 100a and anti-MOMP antibody. Mock-infected and *C. trachomatis*-infected cells were used as negative and positive controls. Each panel is representative of at least three independent experiments.

or IFN- γ significantly reduced the amount of CPAF located in the host cytosol in the majority of infected HeLa (Fig. 3B, C) and primary endocervical cells (Fig. 3E, F). CPAF was distributed in two different patterns in exposed cells. In approximately 89% of infected HeLa cells and 53% of infected primary endocervical cells CPAF was retained within inclusions. In the rest of the cells, CPAF was generally located at the inclusion periphery in the host cytosol. The similarity in the altered CPAF localization observed in the two persistence models suggests that this observation is unlikely to be an artefact.

To further study the localization of CPAF protein with *C. trachomatis* exposed to ampicillin, persistent forms that accumulated at the interfaces of 28–34% and 34–40% Renografin solutions were collected. TEM analysis revealed that the majority of the chlamydial persistent forms in these preparations were intact and all had a lower electron density compared to normal RBs (Fig. 3G, H). While some larger persistent forms (equal or larger than 1 μm in diameter) were present with smaller forms in the 28–34% Renografin sample (Fig. 3G), relatively uniform-sized persistent forms (average $\sim 0.5 \mu\text{m}$ in diameter) were observed in the 34–40% Renografin interface sample (Fig. 3H). Immunoblot analysis with lysates of these samples revealed the presence of CPAF protein corresponding to CPAFc (36 kDa) and its zymogen (~ 65 kDa) (Fig. 3I). In control experiments, CPAF was undetectable in both isolated normal RBs and EBs. These results suggest

that CPAF was associated with the isolated ampicillin-induced persistent forms. Therefore, the observed decrease in host cytosolic CPAF is not due to either decreased synthesis or increased protein degradation in ampicillin-induced *C. trachomatis* persistence.

In infected HeLa cells, CopN-specific immunostaining was not detected until 20 h p.i., and high levels of CopN were observed up to 48 h p.i. (not shown). In normal *C. trachomatis* infection of primary endocervical and HeLa cells at 36 h p.i., the majority of CopN was located inside the inclusion or associated with the inclusion membrane (Fig. 4). This localization is consistent with the observations reported by Fields & Hackstadt (2000) with *C. trachomatis* serovar L2. However, ampicillin exposure altered the staining pattern of CopN, which remained in the inclusion, but displayed a weaker staining signal in the infected cells.

Tarp-specific immunostaining became detectable at 28 h p.i. in infected HeLa cells and was observed at higher levels up to 48 h p.i. (not shown). Tarp was localized within the inclusion and distributed in a punctate pattern in normal infected HeLa (Fig. 4E) and primary endocervical cells (Fig. 4G). In contrast, exposure to ampicillin decreased levels of Tarp, and many inclusions displayed little or no detectable Tarp-specific staining in infected HeLa (Fig. 4F) and primary endocervical cells (Fig. 4H). Unlike CopN and Tarp, the levels and distribution of IncA in the inclusion membrane were not significantly changed upon ampicillin

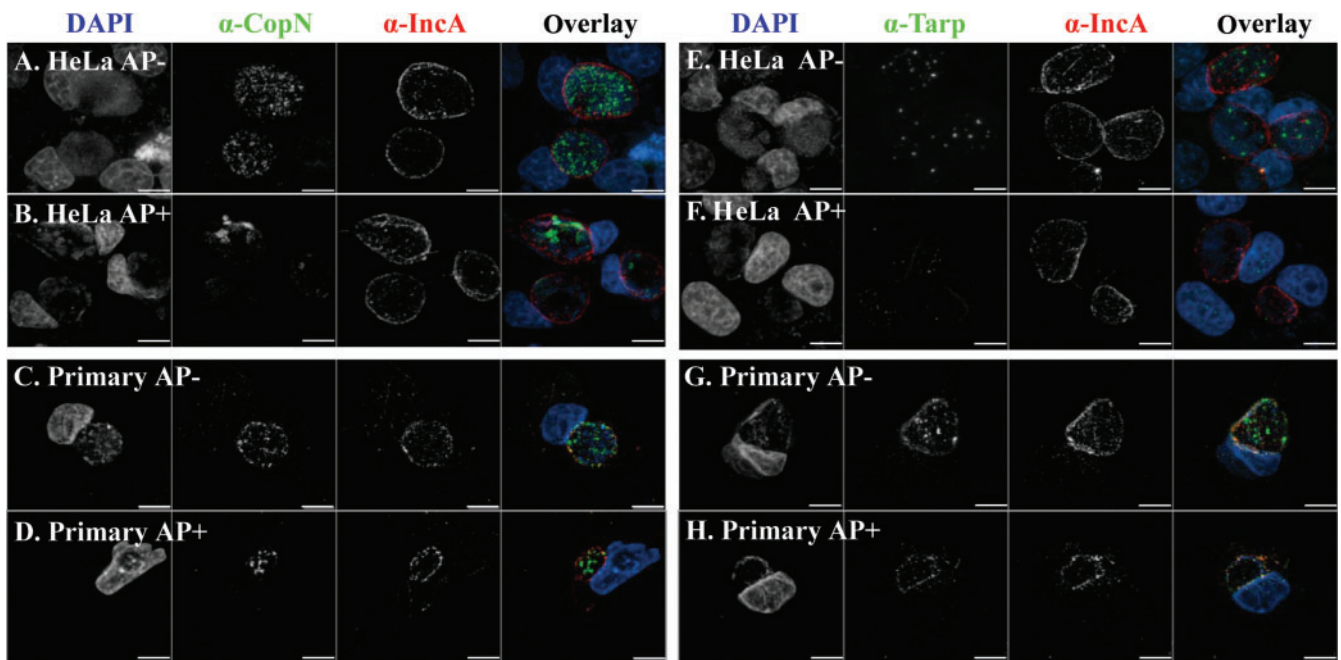


Fig. 4. Analysis of CopN and Tarp proteins in *C. trachomatis*-infected cells. Immunostaining patterns of CopN (left panels) and Tarp (right panels) in infected HeLa (A, B, E, F) and primary human endocervical epithelial cells (C, D, G, H) as indicated. Cells were fixed at 36 h p.i. CopN or Tarp proteins were visualized as green. Host nuclei and bacteria were visualized as blue by counterstaining with DAPI. The inclusion membrane is labelled as red with an anti-IncA antibody. Scale bars, 5 μm . α -, anti-

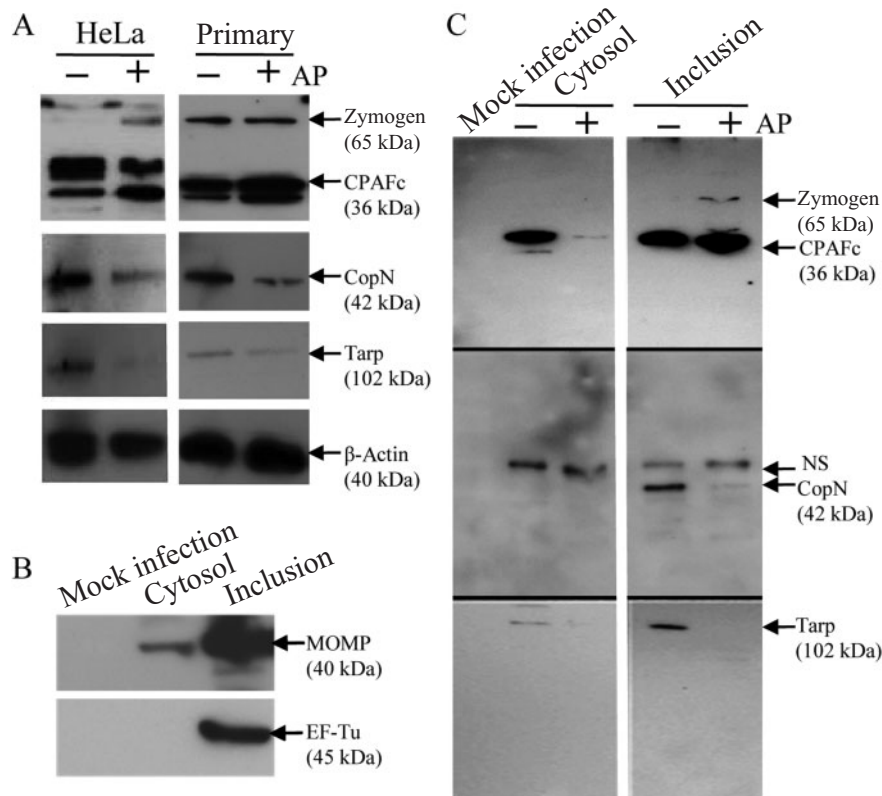


Fig. 5. Quantification of the level and subcellular distribution of *C. trachomatis* CPAF, CopN and Tarp. (A) Levels of CPAF, CopN and Tarp were examined in whole-cell lysates of *C. trachomatis*-infected HeLa and primary human endocervical epithelial cells exposed to ampicillin or under normal growth conditions. Cells were harvested at 36 h p.i. Antibodies specific to chlamydial CPAF, CopN and Tarp were used to visualize relevant proteins. Host β -actin was probed with an anti- β -actin antibody and used to normalize the protein loading amounts. Results are shown of a representative experiment from three HeLa cell experiments, and a representative experiment with primary endocervical epithelial cells derived from one of four donors. (B) Evaluation of cytosol and inclusion fractions from normal infected HeLa cells using immunoprecipitation, followed by immunoblotting with antibodies specific to chlamydial MOMP or EF-Tu. The lysate of mock-infected HeLa cells was used as a negative control. (C) Levels of CPAF, CopN and Tarp proteins in cytosol and inclusion fractions extracted from *C. trachomatis*-infected HeLa cells either under normal growth conditions (AP-) or exposed to ampicillin (AP+), determined by immunoprecipitation followed by immunoblot analysis. A lysate of mock-infected HeLa cells was used as a negative control. The same amounts of *C. trachomatis*-infected HeLa cells were used in the presence of an excess amount of antibody for immunoprecipitation. Results shown are a representative experiment of three independent experiments.

exposure (Fig. 4). Taken together, these results indicate that ampicillin exposure induced different and specific changes in these secretable chlamydial proteins.

***C. trachomatis* responds to ampicillin exposure by selectively altering the expression of secretable proteins in infected cells**

To more precisely identify the mechanisms underlying the observed ampicillin-induced phenotypic changes, whole cellular lysates of ampicillin-exposed and normal *C. trachomatis*-infected cells were assayed by immunoblot analysis for levels of CPAF, Tarp and CopN. Because of the variability in *C. trachomatis* infection in primary endocervical cells from donor to donor, cells derived from four

different donors were examined. In all infected primary endocervical cell cultures, bands corresponding to CPAF (36 kDa) and occasionally its zymogen (~65 kDa) were observed. A representative result is shown in Fig. 5(A), in which the overall levels of CPAF under ampicillin exposure or normal growth conditions were equivalent. A similar result was also observed in HeLa cells. In contrast, levels of Tarp and CopN proteins were clearly decreased in both infected primary endocervical cells and HeLa cells exposed to ampicillin (Fig. 5A).

We then used *C. trachomatis*-infected HeLa cells harvested at 36 h p.i. to separate the host and bacterial protein contents into specific cellular fractions for chlamydial protein distribution analysis. It is difficult to obtain

sufficient primary human endocervical epithelial cells from a single donor for a reproducible cellular fractionation study, and IFA indicates similarity in the localization of CPAF, CopN and Tarp between *C. trachomatis*-infected HeLa and primary endocervical cells (Figs 3 and 4). Cellular fractions defined as cytosol, membrane and inclusion (see Methods) were analysed by immunoprecipitation, followed by immunoblotting. As expected, host β -actin was present in the all three fractions and chlamydial EF-Tu was only present in the inclusion fraction. While chlamydial MOMP was detected at a high level in the inclusion fraction, it was present at a low level in the cytosol fraction (Fig. 5B). Cytosolic MOMP has been described previously and likely associates with the inclusion-membrane vesicles (Giles & Wyrick, 2008). These results suggest that there was no obvious chlamydial cytoplasmic protein contamination in the host-cell fractions.

We next measured the levels of the secretable chlamydial proteins CPAF, CopN and Tarp from cytosol and inclusion fractions using immunoprecipitation, followed by immunoblotting. CPAF was significantly enriched in the cytosol fraction and present at a low level in the inclusion fraction in cultures with normal productive *C. trachomatis* growth (Fig. 5C). In contrast, ampicillin exposure reduced the level of CPAF obtained from the cytosol fraction. Instead, a higher level of CPAF was obtained from the inclusion fraction (Fig. 5C). Nevertheless, the overall quantity of CPAF from both the cytosol and inclusion fractions in ampicillin-exposed cells was approximately equivalent to that found in the infected cells unexposed to ampicillin. These results substantiate our IFA and immunoblot data (Figs 3 and 5A), further suggesting that the total level of CPAF is unchanged, and the CPAF translocation into the host cell cytosol is decreased in ampicillin-exposed cells.

CopN protein was detected only in the inclusion fraction (Fig. 5C). A higher level of CopN was found in normal infected cells, while a reduced level of CopN was observed in ampicillin-exposed cells. The CopN protein band was observed at approximately 42 kDa, close to the predicted molecular mass. A larger band reactive to CopN antibody on the immunoblot was resolved; however, the signal was constant regardless of presence or absence of ampicillin and was absent in mock-infected HeLa cells. Thus, we considered this band non-specific and due to a cross-reaction with an unknown chlamydial protein. A low level of Tarp protein was immunoprecipitated from the cytosol fraction, and a much higher level of Tarp was detected in the inclusion fraction of normal *C. trachomatis*-infected cells (Fig. 5C). Detection of the host-cytosol-localized Tarp at 36 h p.i. was unexpected, as Tarp protein has been reported to be rapidly secreted upon *C. trachomatis*-host cell contact (Clifton *et al.*, 2004). We failed to detect any Tarp signal in samples from ampicillin-exposed HeLa cells. Accordingly, the overall levels of CopN or Tarp from both cytosol and inclusion fractions of ampicillin-exposed cells were substantially lower compared to those from normal infected cells.

To determine whether the levels of protein analysed by immunoblot analysis were in concordance with their mRNA levels, we investigated the transcription of the *copN*, *tarp* and *cpaf* genes in *C. trachomatis*-infected HeLa cells. We used a dual-complex RT-PCR, in which two primer pairs were designed to amplify partial coding regions of *copN*, *tarp* or *cpaf*, as well as *tuf*, which encodes the translation elongation factor EF-Tu. We optimized the conditions that generated compatible amounts of amplicon production with the primers using varied amounts of *C. trachomatis* genomic DNA as templates. No amplified products were observed in the absence of a cDNA synthesis step with *C. trachomatis* RNA as a template. This demonstrated that no detectable DNA contamination was present in the samples. We performed RT-PCR experiments measuring the transcription of *copN*, *tarp* and *cpaf* and then normalized these gene transcript levels to *tuf* transcript levels. The *tuf* gene was chosen as a control for normalization because its product EF-Tu is essential for protein synthesis during all stages of *C. trachomatis* growth (Shen *et al.*, 2000). The transcript ratio was used to compare the relative changes in transcription levels over time. Fig. 6 demonstrates that ampicillin exposure reduced the overall transcription levels of *copN*, *tarp*, *cpaf* and *tuf* in infected cells from 24 to 48 h p.i. compared to those in normal infected cells, consistent with slow-growing bacteria. After normalization to *tuf*, the relative transcript ratio of *cpaf* in ampicillin-exposed cells was equivalent to that in normal infected cells and consistent with the level of total CPAF protein as determined by immunoblot analysis (Fig. 5A). In contrast, relative transcript ratios from *copN* and *tarp* decreased (Fig. 6) in ampicillin-exposed cells harvested at 24, 36 and 48 h p.i. Taken together these results indicate that ampicillin exposure resulted in the selective down-regulation of Tarp and CopN expression, but not CPAF expression, at both the transcriptional and translational level. Thus, the decreased protein levels of CopN and Tarp (Fig. 5C) can be attributed, at least in part, to decreased mRNA levels of *copN* and *tarp*.

DISCUSSION

In this study, we investigated key events that occur during persistence of *C. trachomatis* growth *in vitro*: the developmental cycle, secretion activity, and gene expression. Our work suggests that these events may be involved in the adaptation of *C. trachomatis* to a persistent state in human endocervical epithelial cells.

The value of using primary endocervical cells for biological studies of *C. trachomatis* infection is becoming widely appreciated (Moorman *et al.*, 1986; Rasmussen *et al.*, 1997; Roth *et al.*, 2010). We were able to demonstrate, for the first time, that primary human endocervical epithelial cells cultured *ex vivo* are permissive for *C. trachomatis* infection. Appearance of persistent-like-forms in primary endocervical cell cultures might be the result of the bacteria's ability to adapt to a stricter intracellular niche, as primary

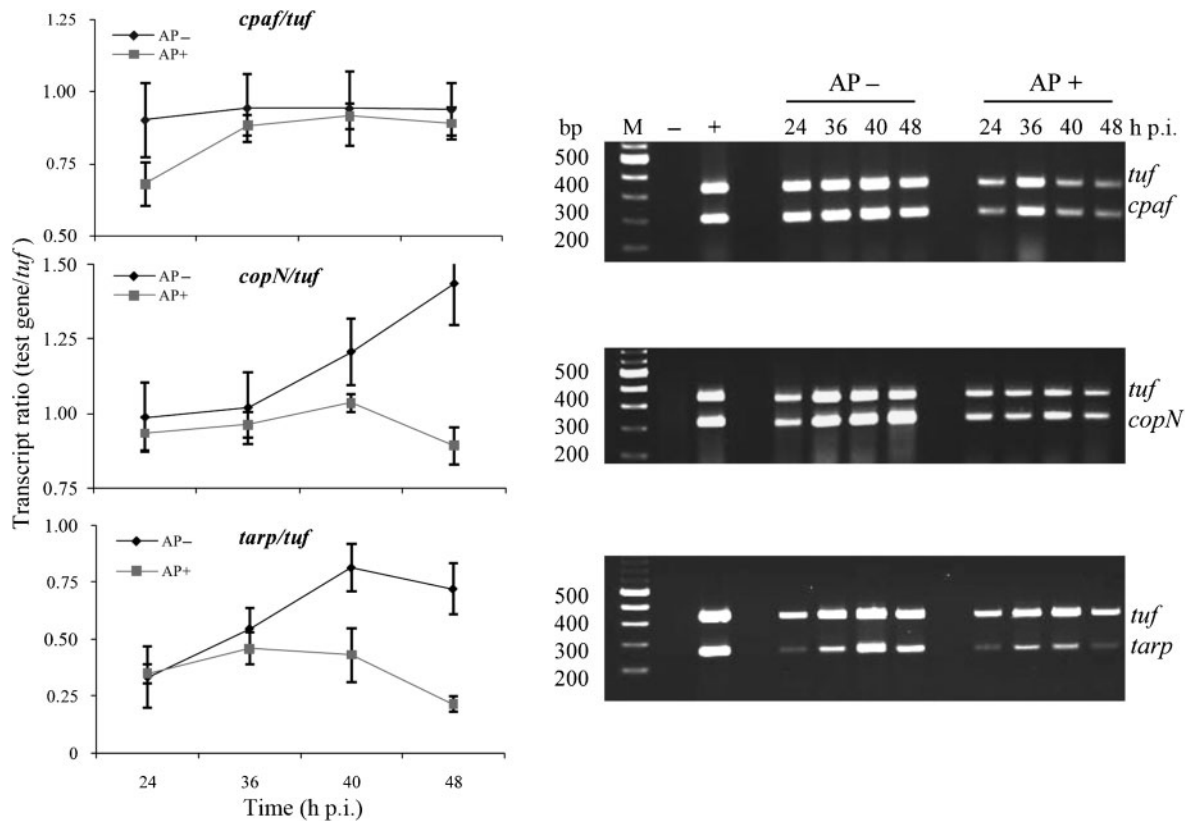


Fig. 6. Transcription analysis of chlamydial genes in *C. trachomatis*-infected HeLa cells by dual-complex RT-PCR. The right panels show ethidium-bromide-stained agarose gels indicating the transcript levels of the *tuf*, *cpaI*, *copN* and *tarp* genes from *C. trachomatis*-infected HeLa cells exposed to ampicillin (AP+) or under normal growth conditions (AP-). Genomic DNA extracted from purified *C. trachomatis* EBs was used as a positive control to demonstrate that in this dual-complex reaction the two primer pairs amplified the targets with similar efficiencies (lane +). RNA isolated from uninfected HeLa cells was used as a negative control (lane -). A fixed quantity of total RNA (host cell RNA + *C. trachomatis* RNA) isolated at the indicated times was treated with DNase I and subjected to RT-PCR. Graphs depicting the mean \pm SD of three independent experiments for each primer set are shown on the left. These show the ratio of *cpaI*, *copN* and *tarp* transcripts to the *tuf* transcripts at different time points p.i. as revealed by the RT-PCR analysis in normal infected cultures or cultures exposed to ampicillin. Note the decrease in transcript levels that occurred in ampicillin-exposed samples due to bacterial growth retardation.

endocervical cells, unlike many transformed cell lines, maintain appropriate expression and regulation of tissue-specific molecules (Fichorova *et al.*, 1997; Rasmussen *et al.*, 1997; Wyrick, 2006). Additionally, this pleiomorphic population of primary endocervical cells at multiple stages of differentiation most likely interacts with *C. trachomatis* differently, resulting in the heterogeneity of infection observed in this study. Therefore, the primary human endocervical epithelial cell model is likely to provide a more accurate representation of *in vivo* infection, and is recommended for validation of work performed in transformed cell lines. One limitation of using primary human endocervical epithelial cells for studies of *C. trachomatis* infection is that it is difficult to grow them in large numbers. In this study, this problem was rectified by the combined use of and comparison with the HeLa infection model. We established that the generation of abnormal persistent forms and the redistribution of

secretable CPAF when *C. trachomatis* is exposed to ampicillin or IFN- γ was not an artefact of transformed cell lines and validated our novel experimental findings observed in HeLa cells.

Our data demonstrate that the vesiculation phenotype is common during productive *C. trachomatis* infection in primary endocervical cells (Fig. 2E). Fewer multivesicular structures are observed in *C. trachomatis*-infected HeLa cells. Although the origin of the multivesicular structures is unclear, it seems likely that they are involved in processes driven by both the bacteria and the host cell. First, they are present only in *C. trachomatis*-infected cells. Second, their localization is closely associated with both the inclusion and the host cytosol. Third, it has been shown that chlamydiae use numerous intracellular trafficking pathways to acquire nutrients from the host cell, and multiple vesicles have been shown to be important organelles in

separating host cell protein and lipid droplets (Beatty, 2006; Cocchiari *et al.*, 2008). Further analyses are needed to determine their origin, dynamics and roles during both productive and persistent chlamydial growth.

It has been previously shown that *C. trachomatis* produces OMVs during the transition from RBs to EBs throughout the developmental cycle (Giles *et al.*, 2006; Stirling & Richmond, 1980). The presence of relatively few and small OMVs in normal productively infected cells and the abundance of OMVs in ampicillin-exposed cells suggest that the formation of OMVs is ampicillin responsive. It is possible that OMV formation is one strategy utilized by *C. trachomatis* in response to stress. In other bacteria, overproduction of OMVs occurs in the presence of stress factors, such as antibiotics (Kadurugamuwa & Beveridge, 1995), phage infection (Loeb, 1974) and envelope stress due to the accumulation of unfolded proteins in the periplasm (McBroom *et al.*, 2006). Increasing evidence indicates that OMVs provide a specific secretory mechanism in Gram-negative pathogens by which diverse virulence factors packaged in OMVs are distantly delivered, allowing pathogens to interact with the host (Amano *et al.*, 2010; Ellis & Kuehn, 2010; Ünal *et al.*, 2010). Giles & Wyrick (2008) reported that vesicles everting from the inclusion membrane can deliver diverse chlamydial antigens into human endometrial HEC-1B epithelial cells. The subsequent impact of chlamydial OMV–host cell interactions on the immune response or disease outcome remains to be elucidated. Since OMV components from other bacteria have proven to be valid vaccine candidates (Amano *et al.*, 2010; Ellis & Kuehn, 2010; Ünal *et al.*, 2010), analysis of immunogenic candidate proteins in chlamydial OMVs may aid in vaccine development for *C. trachomatis*.

The observed decrease of host-cytosol-localized CPAF induced by ampicillin or IFN- γ could result from decreased protein expression, increased degradation or altered transport. Unaltered levels of CPAF transcript and protein in ampicillin-exposed cells suggest the latter is the case. Our data are consistent with previous reports that the transcription (Belland *et al.*, 2003) and protein levels of CPAF (Shaw *et al.*, 2002) are also unchanged in an IFN- γ model of persistence. The finding that ampicillin exposure of *C. trachomatis*-infected primary endocervical epithelial cells alters CPAF localization provides additional support for our observations of CPAF localization in infected HeLa cells. Additionally, we developed the first IFN- γ -mediated persistence model in primary endocervical epithelial cells, which also confirms our findings in HeLa cells as well as findings reported by others (Heuer *et al.*, 2003; Shaw *et al.*, 2002).

In contrast to CPAF, we found that ampicillin induced a decrease in expression of CopN and Tarp. This differs from the findings by Belland *et al.* (2003) in the IFN- γ model of *C. trachomatis* persistence in HeLa cells, in which transcription of the *tarp* gene was downregulated, while *copN* transcription remained unchanged. These results suggest that transcriptional changes of certain chlamydial

genes encoding secretable proteins vary in a stressor-dependent manner, as noted previously (Belland *et al.*, 2003; Klos *et al.*, 2009; Ouellette *et al.*, 2006), perhaps due to differences in the mechanisms by which persistence is induced by different stressors.

Essential to the pathogenesis of *C. trachomatis* is the establishment and maintenance of an intracellular niche and the evasion of host immune responses during infection (Betts *et al.*, 2009). A well-established function of CPAF is the modulation of MHC class I, MHC class II and CD1d expression (Kawana *et al.*, 2007; Zhong *et al.*, 1999, 2000). Because protein function is mostly coupled to protein localization and its levels, altered CPAF localization and decreased levels of T3SS components induced by ampicillin might (directly or indirectly) change the host cell environment. In addition, components of OMVs may also modulate the host signal transduction and inflammatory response (Ellis & Kuehn, 2010; Giles & Wyrick, 2008; Ünal *et al.*, 2010). Alterations of chlamydial protein secretion may allow *C. trachomatis* to achieve intracellular survival upon ampicillin exposure, therefore protecting the bacteria from innate and acquired immunity. Our *in vitro* primary human endocervical epithelial cell model provides a valuable tool to investigate host cell factors that are relevant to the establishment of persistence, as this model most likely retains the appropriate responsiveness to endogenous factors, such as female steroid hormones that can be altered in transformed cervical cell lines (Guseva *et al.*, 2005). While this study has focused on selective chlamydial secretable proteins, a broader proteomic analysis is warranted to elucidate how the overall secretome affects the infection process.

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