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THE OBLIGATE INTRACELLULAR LIFESTYLE

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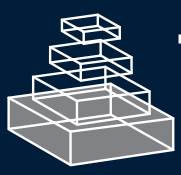

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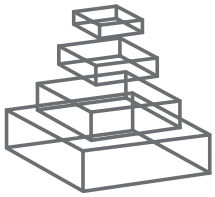

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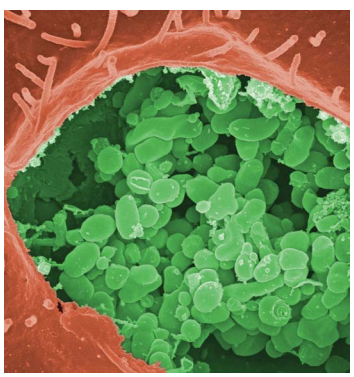
THE OBLIGATE INTRACELLULAR LIFESTYLE

Hosted By
Rey Carabeo, Imperial College London, UK.r.carabeo@imperial.ac.uk
Robert A. Heinzen, NIH/NIAID-RML, USA.rheinzen@niaid.nih.gov
Kenneth A. Fields, University of Miami, USA.kfields@med.miami.edu



Bacterial pathogens whose development is restricted to the interior of eukaryotic cells encounter a unique set of hurdles to be overcome to enable growth. This Research Topic is dedicated to the examination of the unique infection biology associated with this obligate intracellular existence. Distinct approaches to obligate intracellular parasitism are covered by considering a range of molecular, cellular, and immunological mechanisms governing the overall success of pathogens including Chlamydia, Coxiella, Rickettsia, and Ehrlichia. The overall goal is to provide insight into both microbial and host mechanisms that enable colonization of a specialized intracellular niche.

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The obligate intracellular lifestyle

Kenneth A. Fields[1]*, Robert A. Heinzen[2] and Rey Carabeo[3]

[1] *Department of Microbiology and Immunology, University of Miami Miller School of Medicine, Miami, FL, USA*
[2] *Coxiella Pathogenesis Section Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT, USA*
[3] *Division of Cell and Molecular Biology, Centre for Molecular Microbiology and Infection, Imperial College, London, UK*
**Correspondence: kfields@med.miami.edu*

Obligate intracellular bacteria represent consummate parasites, often covertly co-opting host resources to enable development and ultimately transmission to a new host. The overall success of this survival strategy is doubtless derived from co-evolution with respective eukaryotic hosts over hundreds of millions of years. Indeed, many species of obligate intracellular bacteria represent pathogens capable of significant negative impact on worldwide human health. This link to human disease and the fascinating infection biology exhibited by these parasites render them exquisite subjects for investigation. Despite the overarching absolute requirement for growth within eukaryotic cells, this class of bacteria has evolved distinct strategies that enable colonization of diverse tissues, cell types, and even subcellular niches. We have assembled a collection of Opinion, Review, and Primary Research articles that delve into the often unique biology of obligate intracellular bacteria. The reader of this Special Topics Edition will find examples of virulence strategies employed by *Chlamydia*, *Anaplasma*, *Ehrlichia*, and *Rickettsia*. We have also included *Coxiella* in our consideration of obligate intracellular bacteria. Despite the recent development of a host cell-independent culture method (see the review by Beare et al., 2011), *Coxiella burnetii* remains confined to intracellular growth under natural settings. Aspects of covered infection biology include mechanisms of host cell invasion, production, and secretion of anti-host proteins, nutrient acquisition, and host immune response. Unfortunately, the biology that renders this class of microbes so interesting has also often thrown up barriers that complicate investigation. These issues are also touched upon in articles that will hopefully point the way forward.

The ability to invade and gain access to the host cell interior is of obvious importance to obligate intracellular bacteria. However, the precise mechanisms for adherence and invasion remain unresolved for most species. Chan et al. (2010) reviews the current knowledge regarding adherence and invasion by *Rickettsia* spp. and highlights how interference with these events could lead to novel modes of prevention and treatment. This is particularly important since the efficacy of the host immune response can be limited in response to this class of parasites. While the obligate intracellular lifestyle certainly shields pathogens from host defense mechanisms somewhat, some parasite proteins are highly immunogenic. Gall et al. (2011) characterizes *in vitro* and *in vivo* immune responses to chlamydial antigens, and their report raises questions regarding how the immune response contributes to the pathology associated with chlamydial disease.

In contrast to *Rickettsia* spp., intracellular development of *C. burnetii* and *Chlamydia* spp. occurs within a membrane-bound parasitophorous vacuole. Such sequestration presents unique challenges for these organisms and necessitates mechanisms to establish and maintain this unique compartment. Hussain et al. (2011) investigated contributions of eukaryotic factors in formation of the *C. burnetii*-containing vacuole and present evidence that multiple host kinases are essential for vacuole biogenesis. This theme is further explored by Ouellette and Carabeo (2010)who describe an example of how obligate intracellular bacteria intimately interact with and depend on host resources. They demonstrate that recycling rates of transferring-containing vesicles are important for optimal chlamydial growth. Whether this pathway is required to deliver iron was unclear, yet a Methods report from Thompson and Carabeo (2011) illustrates the importance of iron in chlamydial development and establishes a novel method to induce iron starvation during infections with obligate intracellular bacteria.

Similar to the case with their facultative cousins, secretion of host-interactive effector proteins represents a significant mechanism employed by obligate intracellular bacteria to promote virulence through modulation of host cell processes. Therefore, no consideration of obligate intracellular microbiology would be complete without including current views of protein secretion and effector protein function. Betts-Hampikian and Fields (2010) provide a thorough review of the chlamydial type III secretion mechanism and emphasize findings that indicate unique adaptations to the obligate intracellular lifestyle. Stone et al. (2011) extends this discussion in an original research article exploring molecular mechanisms employed to regulate activity of the secretion apparatus. Finally, two articles explore specific effector protein function. Zhong (2011) broadly explores the role of secreted proteases in sculpting the intracellular host environment while simultaneously generating a pool of amino acids for use by parasite, while Broederdorf and Voth (2011) provides an interesting commentary regarding the anti-apoptotic mechanisms of a type IV-secreted *C. burnetii* effector protein.

Investigating the infection biology of obligate intracellular bacteria is often a complicated process. First, psychological barriers can exist that make researchers hesitate to even carry out research. For example, Wolf (2011) describes the dilemma currently limiting interest regarding the pathogenesis of *Chlamydia pneumoniae*. However, the lack of tractable genetic system represents perhaps the greatest barrier confounding significant progress in obligate intracellular biology. In the past, rigorous proteomic studies have helped drive progress in the absence of mutant generation. For example, the proteomic analyses of *Anaplasma* and *Ehrlichia* performed by Lin et al. (2011) illustrate how these studies can provide useful insight. Happily, the future now seems

bright for some degree of genetic manipulation. Beare et al. (2011) provide a thoughtful review of factors that have confounded efforts in the past and summarize exciting advances where real progress has been made in genetic analyses of obligate intracellular bacteria. Significantly, advances have also come been made in historically intractable *Chlamydia* system where it is now possible to generate targeted mutations in chlamydial genes. As the sampling of articles in this Special Topics issue illustrates, the biology and pathogenesis of obligate intracellular bacteria represents a fertile area of investigation that is likely poised to expand rapidly in the future.

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Advances in genetic manipulation of obligate intracellular bacterial pathogens

Paul A. Beare[1], Kelsi M. Sandoz[2], Anders Omsland[1], Daniel D. Rockey[2] and Robert A. Heinzen[1]*

[1] *Coxiella Pathogenesis Section, Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT, USA*

[2] *Department of Biomedical Sciences, Oregon State University, Corvallis, OR, USA*

Edited by:
Rey Carabeo, Imperial College London, UK

Reviewed by:
Mikhail A. Gavrilin, Ohio State University, USA
Andres Vazquez-Torres, University of Colorado Medical School, USA

***Correspondence:**
Robert A. Heinzen, Rocky Mountain Laboratories, Laboratory of Intracellular Parasites, 903 South Fourth Street, Hamilton, MT 59840, USA.
e-mail: rheinzen@niaid.nih.gov

Infections by obligate intracellular bacterial pathogens result in significant morbidity and mortality worldwide. These bacteria include *Chlamydia* spp., which causes millions of cases of sexually transmitted disease and blinding trachoma annually, and members of the α-proteobacterial genera *Anaplasma*, *Ehrlichia*, *Orientia*, and *Rickettsia*, agents of serious human illnesses including epidemic typhus. *Coxiella burnetii*, the agent of human Q fever, has also been considered a prototypical obligate intracellular bacterium, but recent host cell-free (axenic) growth has rescued it from obligatism. The historic genetic intractability of obligate intracellular bacteria has severely limited molecular dissection of their unique lifestyles and virulence factors involved in pathogenesis. Host cell restricted growth is a significant barrier to genetic transformation that can make simple procedures for free-living bacteria, such as cloning, exceedingly difficult. Low transformation efficiency requiring long-term culture in host cells to expand small transformant populations is another obstacle. Despite numerous technical limitations, the last decade has witnessed significant gains in genetic manipulation of obligate intracellular bacteria including allelic exchange. Continued development of genetic tools should soon enable routine mutation and complementation strategies for virulence factor discovery and stimulate renewed interest in these refractory pathogens. In this review, we discuss the technical challenges associated with genetic transformation of obligate intracellular bacteria and highlight advances made with individual genera.

Keywords: transposon mutagenesis, electroporation, antibiotic selection, allelic exchange, genetic transformation, virulence factor, shuttle vector, complementation

INTRODUCTION

Obligate intracellular bacterial pathogens are an understudied but significant group of human disease agents. These bacteria are thought to have emerged from ancestral non-pathogens through a pathoadaptive evolutionary process that involves significant genome reduction (Pallen and Wren, 2007). Ongoing gene loss in obligate intracellular bacteria is indicated by the presence of pseudogenes whose functions are often compensated for by the host cell. Some obligate intracellular bacteria, such as *Rickettsia prowazekii* and *Chlamydia trachomatis*, appear in the final stages of host cell adaptation, having cleared most pseudogenes from their respective genomes (Andersson and Andersson, 1999). Relative to these bacteria, *Coxiella burnetii* has a sophisticated genome with central metabolic pathways largely intact, suggesting a more recent adaptation to an obligate intracellular lifestyle (Seshadri et al., 2003).

Despite dramatically reduced genomes relative to most free-living bacterial pathogens, obligate intracellular bacterial pathogens still retain potent pathogenetic potential that can manifest in infections ranging from asymptomatic to fulminating and deadly (Walker, 1989; Maurin and Raoult, 1999). Unfortunately, the historic lack of genetic tools for obligates has severely limited molecular dissection of mechanisms associated with intracellular parasitism and animal pathogenesis. Many genes encoding putative virulence factors have been revealed by pathogen genome sequences (Andersson et al., 1998; Seshadri et al., 2003). However, because methods for site-specific gene inactivation and complementation are lacking, molecular Koch's postulates (Falkow, 2004) have been impossible to fulfill for these genes. Consequently, gene function and regulation have often been explored using heterologous expression in surrogate hosts (Whitworth et al., 2005; Raghavan et al., 2008; Voth et al., 2009).

In this review, we discuss the experimental hurdles associated with developing genetic transformation systems for obligate intracellular bacteria and review the genetic tools that are currently available.

TECHNICAL CONSIDERATIONS IN TRANSFORMING OBLIGATE INTRACELLULAR BACTERIA

A pathogen's obligate reliance on a eukaryotic host cell for growth complicates several steps in genetic transformation that are easily conducted with free-living bacteria. Nonetheless, by employing tenacity and attention to detail, several investigators have overcome technical hurdles to establish at least rudimentary genetic systems for most pathogenic obligate intracellular bacteria. In this section, we highlight the special experimental considerations associated with genetic transformation systems of these bacteria.

BACTERIAL PURIFICATION

Before any genetic transformation procedure, obligate intracellular bacteria must be purified to some extent from host cells and concentrated to high density in a viable form. Depending on the degree of purity, the procedure can involve several centrifugation steps that take nearly a full day to complete (Shannon and Heinzen, 2007). For organisms that grow to low density in host cells, such as spotted fever group (SFG) rickettsia, yields can be poor and allow for only a few electroporation experiments (Kleba et al., 2010). To ensure utmost viability, some obligate intracellular bacteria are electroporated immediately after purification (Qin et al., 2004), thereby eliminating the convenience of storing purified bacteria for subsequent transformation experiments. Several low ionic strength electroporation buffers have been used, ranging from distilled water (Binet and Maurelli, 2009) to buffers containing osmoprotectants such as sucrose and glycerol (Beare et al., 2009). Organisms are washed several times in buffers and resuspended at high density (approx. 10^{10} bacteria per ml) prior to electroporation.

A consideration when purifying obligate intracellular bacteria for transformation experiments is that many display developmental forms that may be differentially infective and/or receptive to electroporation. For example, the large reticulate cell (RC) of *Anaplasma phagocytophilum* may be more amenable to electroporation than the smaller dense-cored cell (DC) with its characteristic condensed chromatin. However, the RC is poorly infective relative to the DC (Troese and Carlyon, 2009). A similar and more extreme example involves reticulate bodies (RB) of chlamydia that may be quite receptive to electroporation but are difficult to purify and considered non-infectious (Bavoil et al., 2000). Large cell variant (LCV) and small cell variant (SCV) development forms of *C. burnetii* appear equally infectious for host cells (Coleman et al., 2004). However, because the permissiveness of SCV and LCV to electroporation is unknown, bacteria used in transformation experiments are purified when host cells contain roughly equal numbers of cell forms (Beare et al., 2009).

ANTIBIOTIC SELECTION AND CONSTRUCT OPTIMIZATION

Thus far, positive selection of transformed obligate intracellular bacteria has been conducted exclusively by selecting for antibiotic resistance. Restrictions based on antibiotic clinical efficacy in treating human infections significantly reduces the set of antibiotic resistance genes suitable for transformation studies. Furthermore, in the United States, the Centers for Disease Control and Prevention, Division of Select Agents and Toxins, ultimately approves the use of antibiotic resistance genes in select agent pathogens. *R. prowazekii*, *Rickettsia rickettsii*, and virulent phase I strains of *C. burnetii* fall into this category (Atlas, 2003). Work with these organisms also requires stringent biosafety level 3 procedures.

The minimal inhibitory concentrations (MIC) of approved antibiotics must first be established in a relevant host cell model system. Complicating the establishment of MICs are issues related to permeability and subcellular pharmacological activity. With the exception of cells infected with cytoplasmically localized *Rickettsia* or *Orientia* spp., antibiotics used in selection must permeate at least two host cell lipid bilayers: the plasma membrane and the membrane of the pathogen-occupied vacuole. To overcome these diffusion barriers, the concentration of antibiotics required for selection may be several fold higher than typically used with free-living bacteria. A high MIC may be toxic to host cells. For instance, high levels of chloramphenicol can inhibit mitochondrial function (Li et al., 2010). Moreover, the microenvironment of intracellular compartments may inhibit antibiotic activity. For example, the acidic parasitophorous vacuole of *C. burnetii* clearly inhibits the bactericidal effect of certain antibiotics (Maurin et al., 1992). Raising vacuolar pH with alkalizing agents, such as hydroxychloroquine, can dramatically increase antibiotic killing of *C. burnetii* (Maurin et al., 1992). Indeed, long-term combination doxycycline/hydroxychloroquine therapy is now recommended for treatment of chronic Q fever endocarditis (Maurin and Raoult, 1999). Finally, high rates of spontaneous mutation to resistance is a problem with some pathogen–antibiotic pairs such as *C. burnetii*–ampicillin (Suhan et al., 1996) and *R. prowazekii*–rifampin (Rachek et al., 1998). Expansion of non-transformed resistant mutants is exacerbated by the several week selection procedures used in many transformation protocols for obligates.

Constructs used in transformation generally have antibiotic resistance genes and supplementary screenable markers, such as genes encoding fluorescent proteins, under control of a characterized pathogen promoter. Again, developmental biology needs to be considered when picking a promoter to ensure constitutive expression of marker genes throughout the infectious cycle of a pathogen. Optimal gene expression may require use of chemically synthesized genes that more closely match the codon usage of a particular organism. This is a particular issue with pathogens having unusual genomic G + C content, such as *Rickettsia* spp. (Qin et al., 2004). Finally, active pathogen restriction/modification systems may significantly lower transformation frequency. Propagation of transformation constructs in methylation defective *E. coli* strains may alleviate this problem (Binet and Maurelli, 2009).

INTRODUCTION OF DNA

Electroporation has been universally effective in introducing DNA into obligate intracellular bacteria. Because of their relatively small particle size, high electrical field strengths (e.g., 16–24 kV/cm) are generally used, with organisms typically maintaining high viability after the procedure. Indeed, two pulses of 16 kV/cm were found optimal for DNA uptake by the ~0.3 μm in diameter elementary bodies (EB) of *Chlamydia psittaci* (Binet and Maurelli, 2009). Also facilitating DNA uptake is the use of relatively large amounts of DNA (e.g., 5–10 μg; Rachek et al., 1998; Beare et al., 2009; Binet and Maurelli, 2009). Prior to infection of host cells, DNaseI protection assays can be conducted to confirm uptake of target DNA by microorganisms. Electroporated organisms are treated with DNaseI to remove extracellular plasmid, and the plasmid DNA protected by pathogen uptake is detected by Q-PCR (Tam et al., 1994; Rachek et al., 1998; Binet and Maurelli, 2009).

SCORING AND EXPANSION OF TRANSFORMANTS

Obligate intracellular bacteria have slow generation times relative to most free-living bacteria. For example, *C. burnetii* has a generation time of approximately 11 h during exponential phase in Vero cells (Coleman et al., 2004). Thus, to allow adequate expression of resistance markers, antibiotics are generally added to tissue culture media at 12–24 h post-infection with electroporated organisms (Rachek

et al., 1998; Baldridge et al., 2005; Felsheim et al., 2006; Beare et al., 2009; Binet and Maurelli, 2009). Delayed addition of antibiotic may also be required to allow differentiation and metabolic activation of developmental forms (Bavoil et al., 2000).

A preliminary score of successful transformation is obvious growth of organisms in tissue culture containing an inhibitory concentration of antibiotic. Low transformation frequencies combined with slow growth often require incubations over several weeks to expand transformants to a level detectable by microscopy (Rachek et al., 1998; Baldridge et al., 2005, 2010b; Beare et al., 2009; Felsheim et al., 2010). Expansion of organisms that do not actively lyse host cells, such as *C. burnetii*, is aided by sequential rounds of infection, mechanical host cell lysis, and re-infection of fresh host cells (Beare et al., 2009). Transformants of *R. rickettsii* and *C. psittaci* have been detected in 7 and 10 days, respectively, by using plaque assays (described below; Binet and Maurelli, 2009; Kleba et al., 2010; Clark et al., 2011).

Because spontaneous mutation to antibiotic resistance can result in significant background numbers of non-transformed bacteria (Suhan et al., 1996; Rachek et al., 1998), additional screens are required to confirm genetic transformation. Molecular typing of transformants, either as clones or pools, by Southern blotting is arguably the gold standard and most commonly used method for confirming stable maintenance of target DNA (Suhan et al., 1996; Rachek et al., 1998; Qin et al., 2004; Felsheim et al., 2006; Baldridge et al., 2007a, 2010b; Liu et al., 2007; Beare et al., 2009; Binet and Maurelli, 2009; Driskell et al., 2009; Chen et al., 2010; Kleba et al., 2010). Often complementing Southern blotting is rescue cloning of transposon (Tn)-encoded antibiotic resistance genes and sequencing to map integration sites (Qin et al., 2004; Baldridge et al., 2005, 2007a, 2010b; Felsheim et al., 2006, 2010; Liu et al., 2007; Beare et al., 2009). Direct sequencing of transformant chromosomal DNA has also mapped Tn integration sites (Kleba et al., 2010). Sequencing of recombination sites using PCR and primers flanking target genes has confirmed allelic exchange experiments (Rachek et al., 1998; Binet and Maurelli, 2009). Assessment of transformation by PCR with primers specific to only transformation DNA should be avoided as false positive amplicons can arise from the persistence of free transformation DNA in cell culture (P. A. Beare and R. A. Heinzen, unpublished data).

Direct detection of reporter/antibiotic resistance gene expression has frequently supplemented genome analysis in scoring transformation. A commonly used method is microscopic visualization of green or red fluorescent proteins encoded by introduced transgenes (Lukacova et al., 1999; Troyer et al., 1999; Renesto et al., 2002; Baldridge et al., 2005, 2010b; Felsheim et al., 2006, 2010; Liu et al., 2007; Beare et al., 2009; Chen et al., 2010). RT-PCR, northern blotting and immunoblotting have been employed by several investigators to confirm expression of fluorescent and antibiotic resistance proteins (Suhan and Thompson, 2000; Baldridge et al., 2005, 2010b; Felsheim et al., 2010).

CLONING OF TRANSFORMANTS

Generation of clones from a mixed population of transformants is essential for downstream phenotypic analyses. Cloning derives organisms with single mutational events and eliminates potential viable but non-transformed carryover bacteria that, depending on the experiment, may allow removal of antibiotic selection. Cloning of free-living bacteria, but not obligate intracellular bacteria, is easily done by colony formation on agar plates. A convenient method for some obligate intracellular bacteria is the plaque assay. This procedure relies on pathogen destruction of a localized region of the host cell monolayer that is visible to the naked eye. Monolayer plaques are picked through an agarose overlay and the harvested clonal populations of bacteria further expanded. The assay is generally applicable to organisms with active mechanisms of cell-to-cell spread and/or host cell lysis, such as SFG rickettsia (Wike et al., 1972) and some *Chlamydia* spp. (Matsumoto et al., 1998). Indeed, the assay was recently employed to clone transformants of *R. rickettsii* (Kleba et al., 2010) and *C. psittaci* (Binet and Maurelli, 2009). An alternate method of cloning involves extraction of bacteria from individual infected cells by micromanipulation (Beare et al., 2007). Infections conducted at a low multiplicity of infection (e.g., 0.1) initially result in cells that contain the biological equivalent of a very small bacterial colony. A micromanipulator is used to place a glass micropipette, attached to a hydraulically driven microinjector, directly into an isolated infected cell. The clonal bacterial contents are then removed by running the microinjector in reverse. This procedure is applicable to pathogens where infected monolayer-forming host cells are easily identifiable by light microscopy. The method was recently used to clone a Tn mutant of *C. burnetii* (Beare et al., 2009). Finally, transformant clones of *C. burnetii* (Suhan et al., 1996) and *R. prowazekii* (Rachek et al., 2000; Qin et al., 2004) have been derived by the tedious and time-consuming method of end-point limiting dilution.

SUCCESSES IN TRANSFORMATION OF OBLIGATE INTRACELLULAR BACTERIAL PATHOGENS

Despite considerable technical barriers, significant advances in genetic manipulation of obligate intracellular bacteria have occurred in the last decade. These include (1) Tn mutagenesis, which has identified a key virulence determinant in rickettsia and allowed generation of fluorescent bacteria useful in host–pathogen interaction studies, (2) discovery of a *C. burnetii* shuttle vector used in identifying secreted effector proteins, and (3) the first successful site-specific inactivation of a virulence gene by allelic exchange. Achievements specific to bacterial genera are outlined below.

RICKETTSIA SPP.

Efforts with *R. prowazekii*, the cause of epidemic typhus, have led the way in genetic manipulation of obligate intracellular bacteria. The organism was originally transformed to rifampin resistance by using a portion of the *R. prowazekii rpoB* gene from a strain containing a mutation conferring resistance to rifampin (Rachek et al., 1998). Following electroporation with a ColE1-based suicide plasmid carrying the allele, recombination occurred between the plasmid and chromosomal *rpoB* locus to result in allelic exchange and genetic transformation to rifampin resistance (Rachek et al., 1998).

The described rifampin resistance allele is recessive in a merodiploid strain containing a wild-type gene, thereby limiting its use in *R. prowazekii* as a selectable marker (Wood and Azad, 2000). Therefore, two alternate methods of positive selection were tested that employed genes that are dominant by producing enzymes that

inactivate antibiotics. The first system utilized *ereB*, which encodes an esterase that hydrolyzes the lactone ring of erythromycin (Rachek et al., 2000). Successful transformation to erythromycin resistance resulted from chromosomal integration of a suicide plasmid carrying *ereB* and rickettsial *gltA*, encoding citrate synthase. Homologous recombination by a single crossover event was mediated by *gltA* to result in two chromosomal copies of *gltA* flanking heterologous *ereB* (Rachek et al., 2000). The second system used *arr-2* which encodes an enzyme that inactivates rifampin by ADP-ribosylation (Qin et al., 2004; Liu et al., 2007). For optimal expression, *arr-2* codons were engineered to mimic rickettsial codon usage and cloned downstream from a strong *R. prowazekii* ribosomal subunit promoter ($rpsL^P$). The *arr-2* cassette was incorporated into two Tn systems that were used to randomly mutagenize *R. prowazekii*. The first system employed an Epicentre EZ::Tn5 transposon system (Qin et al., 2004). Experiments inconsistently yielded transformants, and when successful, only a few unique Tn insertion sites were identified (Qin et al., 2004). Inconsistency in generating active transposome complexes (i.e., Tn plus transposase) was speculated as contributing to low transformation efficiency (Qin et al., 2004; Liu et al., 2007). Seeking a more robust system of random mutagenesis, a *mariner*-based *Himar1* Tn system was tested (Lampe et al., 1999; Liu et al., 2007). Transposition of *Himar1* only requires expression of the *Himar1* transposase and no additional host factors (Lampe et al., 1999), and was previously used by Felsheim et al. (2006) in successful Tn mutagenesis of the obligate intracellular bacterium *A. phagocytophilum*. *R. prowazekii* was electroporated with a suicide plasmid (pMW1650) encoding *Himar1* with an integral *arr-2* cassette, a green fluorescent protein (GFP) gene driven by the strong rickettsial outer membrane protein A (OmpA) promoter (Baldridge et al., 2005), and a ColE1 origin of replication for rescue cloning. Transformation resulted in green fluorescent rickettsiae and numerous transposition events. The improved transformation efficiency of *Himar1* over the transposome system was attributed, in part, to the 2-bp TA recognition sequence of *Himar1* and the A + T richness of rickettsiae (~71%).

Transformation using pMW1650 has recently provided novel information on virulence mechanisms of *R. rickettsii*, the cause of Rocky Mountain spotted fever. In one study, transformation yielded an unusually small rifampin-resistant plaque (Kleba et al., 2010). Unlike typhus group rickettsia, SFG rickettsia readily form plaques in epithelial cell and fibroblast monolayers (Wike et al., 1972). Mapping of the *Himar1* insertion site of this clone revealed that *sca2*, encoding an autotransporter protein, had been insertionally inactivated. The mutation and small plaque phenotype correlated with defective actin-based motility, a mechanism used by SFG rickettsia for cell-to-cell spread (**Figure 1**; Heinzen et al., 1993). Sca2 has since been defined as a bacterial actin nucleator that functionally mimics eukaryotic formin proteins (Haglund et al., 2010). A definitive virulence role for *sca2* was demonstrated by showing attenuated virulence of the mutant in a guinea pig challenge model (Kleba et al., 2010). A second study elucidated the mechanism of plaque clarity in SFG rickettsia and resolved a running debate on whether the phenotype is associated with virulence. Lytic strains that produce clear plaques have historically been considered more virulent than non-lytic strains that form opaque or "turbid" plaques (Hackstadt, 1996). Genome sequencing of spontaneously arising non-lytic and lytic variants of normally lytic and non-lytic strains, respectively, revealed that lytic strains of *R. rickettsii* have mutations in a gene annotated as *relA/spoT*-like that are predicted to disrupt protein function. Transformation of a lytic variant with a pMW1650 construct that contains *Himar1* with a wild-type version of *relA/spoT*, complemented the mutant to result in a non-lytic, turbid plaque phenotype. Thus, RelA/SpoT activity is responsible for the turbid plaque phenotype, presumably manifested by the protein's putative role in rickettsial alarmone physiology. Interestingly, isogenic lytic and non-lytic strains were equally virulent for guinea pigs, indicating plaque clarity is not a correlate of virulence in SFG rickettsia (Clark et al., 2011).

Finally, in the first and currently only example of targeted disruption of a putative virulence gene encoded by an obligate intracellular bacterium, the *R. prowazekii pld* gene, encoding phospholipase D, was disrupted by a double crossover event with a linear 2698 bp DNA fragment containing *pld* disrupted with the *arr-2* cassette (Driskell et al., 2009). Like the *R. rickettsii sca2* mutant, the *pld* mutant showed attenuated virulence in a guinea pig challenge model (Driskell et al., 2009).

Achievements with *R. prowazekii* were followed by transformation of non-pathogenic *Rickettsia monacensis* and *Rickettsia montanensis* to chloramphenicol resistance using the EZ::Tn5 system

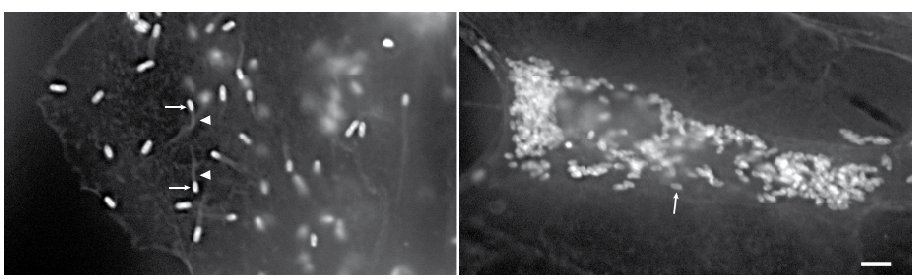

FIGURE 1 | A *Himar1* insertion in *R. rickettsii sca2* eliminates actin-based motility. The right micrograph shows wild-type *R. rickettsii* (arrows) with typical filamentous actin tails (arrowhead). The left micrograph depicts a small plaque-forming mutant of *R. rickettsii* with a *Himar1* insertion in *sca2,* which encodes an autotransporter protein (Kleba et al., 2010). The mutant rickettsia (arrow) lack actin tails. Rickettsia were stained by immunofluorescence using a specific monoclonal antibody and filamentous actin was stained with Alex Fluor 598 phalloidin. Bar, 3 μm. (Micrographs courtesy of Ted Hackstadt, Rocky Mountain Laboratories).

(Baldridge et al., 2005, 2010b). In transformation of *R. monacensis*, the Tn carried genes encoding chloramphenicol acetyltransferase (CAT) and GFP independently driven by the rickettsial OmpA promoter. Following extensive tissue culture passage, a clonal population of green fluorescent rickettsia with a single Tn insertion was obtained. The transformant proved useful in visualizing infection dynamics of *R. monacensis* in ticks (Baldridge et al., 2007b). Mapping of a Tn5 insertion site in a later transformation experiment revealed a hitherto unknown 23.5 kb low copy-number plasmid in *R. monacensis* (Baldridge et al., 2007a). Termed pRM, the plasmid is highly related to pRF, 39.2 kb plasmid previously identified in *Rickettsia felis* by genome sequencing (Ogata et al., 2005). Related plasmids have now been discovered in multiple *Rickettsia* spp. (Baldridge et al., 2010a). A valuable shuttle vector for rickettsial transformation could be derived by subcloning regions necessary for pRM/pRF replication into a standard *E. coli* cloning vector. For transformation of *R. montanensis,* the EZ::Tn5 construct was altered to contain GFP and CAT genes under control of the rickettsial outer membrane protein B (OmpB) promoter (Baldridge et al., 2010a). Because OmpB is more abundant than OmpA in SFG rickettsia (Policastro and Hackstadt, 1994), enhanced expression of GFP was anticipated. Again, a clone containing a single Tn insertion was isolated after cell culture passage; however, GFP expression was poor, leading the authors to conclude that the *ompA* promoter is a better choice for driving expression of foreign genes in SFG rickettsia (Baldridge et al., 2010a).

Single reports have been published on transformation of *Rickettsia typhi* (Troyer et al., 1999) and *Rickettsia conorii* (Renesto et al., 2002), the etiologic agents of murine typhus and Mediterranean spotted fever, respectively. Both studies employed suicide vectors containing *gfp* cloned immediately downstream of the 3′ end of a wild-type version of the pathogen's *rpoB* gene. PCR demonstrated homologous recombination between chromosomal and plasmid *rpoB* sequences, and flow cytometry showed elevated levels of green fluorescence in cells infected with transformant populations. While demonstrating the feasibility of transforming these rickettsial species, the lack of a selectable marker limits the utility of the described transformation strategies.

A. PHAGOCYTOPHILUM AND *ANAPLASMA MARGINALE*

Anaplasma phagocytophilum and *A. marginale,* which cause human and bovine anaplasmosis, respectively, have been transformed with *Himar1* (Felsheim et al., 2006, 2010). In these cases, the *Himar1* transposase and Tn were carried on separate suicide plasmids, thereby eliminating promiscuous transposition events that can occur during plasmid propagation in *E. coli* when both elements are carried on a single plasmid (P. A. Beare, personal observation). Expression of Tn-encoded GFP and spectinomycin resistance genes was driven by the *A. marginale tr* promoter (Barbet et al., 2005) as single transcriptional unit. Transposase expression was also directed by the *tr* promoter. Bright green fluorescent bacteria resulted from co-transformation with both suicide plasmids. Multiple Tn integration sites were mapped in *A. phagocytophilum* transformants (Felsheim et al., 2006). Conversely, after 2 months of antibiotic selection, only a single Tn insertion site was identified in *A. marginale*. Surprisingly, in this instance, transformation did not result from Tn transposition but rather from integration of plasmid DNA by single crossover at the *tr* promoter region (Felsheim et al., 2010). Nonetheless, this transformant has proven useful in monitoring transmission of *A. marginale* between cattle and ticks (Noh et al., 2011).

COXIELLA BURNETII

The most sophisticated genetic tools are currently available for *C. burnetii,* which incidentally, was the first obligate intracellular bacterium to be stably genetically transformed in 1996 (Suhan et al., 1996). As discussed in more detail below, recent advances in *C. burnetii* genetic transformation have been dramatically aided by the recent discovery of a medium that supports host cell-free (axenic) growth of the organism (Omsland et al., 2009, 2011).

The first successful genetic transformation of *C. burnetii* used a chimeric plasmid termed pSKO(+)1000 comprised of a previously defined *C. burnetii* 5.8 kb autonomous replication sequence (*ars*) (Suhan et al., 1994) and the *E. coli* cloning vector pBluescript, which carries a β-lactamase (*blaM*) gene and a ColE1 *ori* (Suhan et al., 1996). Ampicillin-resistant *C. burnetii* were recovered from host cells after 2–3 months of selection. Southern blotting of transformant DNA with a *blaM* probe showed both chromosomal integration and *ars*-dependent autonomous replication of pSKO(+)1000. Integration occurred via homologous recombination between the plasmid *ars* and the corresponding region in the *C. burnetii* chromosome (Suhan et al., 1996). The lengthy selection process also yielded ampicillin-resistant organisms without *blaM*, indicating spontaneous mutation to antibiotic resistance had occurred. In a subsequent transformation report, pSKO(+)1000 was modified to contain a GFP reporter gene that, like the *blaM* gene, was driven by an *E. coli* promoter (Lukacova et al., 1999). Weakly green fluorescent organisms were shown, but molecular data confirming transformation was not provided (Lukacova et al., 1999).

Over a decade elapsed before the next successful transformation of *C. burnetii*, an accomplishment that employed the *Himar1* Tn (Beare et al., 2009). Similar to the *Anaplasma* spp. *Himar1* system (Felsheim et al., 2006), a two-plasmid system was used with a pathogen promoter, in this case 1169^{P}, driving expression of transposase, CAT, and mCherry red fluorescent protein genes. CBU1169 encodes the small heat shock protein Hsp20 and is constitutively expressed at a moderate level throughout the *C. burnetii* infectious cycle (P. A. Beare and R. A. Heinzen, unpublished data). After 5 weeks of incubation in the presence of antibiotic and multiple passages, Vero cells infected with *C. burnetii* co-electroporated with both *Himar1* plasmids displayed obvious bacteria-filled intracellular vacuoles. Subsequent rescue cloning of the ColE1 *ori* revealed 35 unique Tn insertion sites, with two located in the 37.4-kb QpH1 plasmid. A transformant termed B2c was cloned by micromanipulation (Beare et al., 2007), expanded for 5 weeks in cell culture, and shown to contain a Tn insertion in *ftsZ*, a gene required for bacterial cell division. The FtsZ mutant grew in long filaments having incomplete division septae and represented the first example of a clonal population of *C. burnetii* harboring a defined gene mutation generated by genetic transformation.

Random mutagenesis of *C. burnetii* using a host cell-based transformation protocol was a significant advance in genetic manipulation of the organism. However, the lengthy selection and clonal

expansion processes severely limits the number of clones that can be phenotyped as well as the testing of new transformation technologies. Moreover, obligatism eliminates Tn mutants that cannot infect and subsequently grow in host cells, thereby precluding identification of many genes required for virulence. However, the recent breakthrough in axenic growth of *C. burnetii* has now removed the substantial experimental constraints associated with an obligate intracellular lifestyle (Omsland et al., 2009, 2011). An axenic growth medium called acidified citrate cysteine medium (ACCM) was developed that supports roughly 3 logs ($\log_{10}$) of growth of *C. burnetii* over 6 days in a microaerobic environment. Importantly, axenically grown organisms plate with high efficiency in semi-solid ACCM agarose and the minute colonies that arise contain clonal populations (Omsland et al., 2009, 2011).

An entirely axenic transformation protocol is now accelerating development of *C. burnetii* genetic tools. Bacteria for electroporation are grown in ACCM for 6 days to early stationary phase. Following electroporation, organisms are incubated in ACCM for 24 h prior to addition of antibiotic. Three days later, organisms are plated in semi-solid ACCM agarose containing antibiotic and plates incubated for 6 days to allow colony formation. Colonies are picked, added to ACCM, and cultures incubated for 6 days to expand the clone. Thus, expanded transformant clones can now be derived in 2–3 weeks using ACCM as opposed to 2–3 months using cell culture. Moreover, both CAT and Kan genes work well as selectable markers in ACCM, allowing two transformation procedures in a single strain (Omsland et al., 2011).

Using ACCM, an optimized *Himar1*-based Tn mutagenesis system was developed for *C. burnetii*. *1169*^{P}-driven expression of CAT and mCherry as a single transcriptional unit in the original *Himar1* system resulted in sufficient chloramphenicol resistance, but disappointing mCherry fluorescence (Beare et al., 2009). Therefore, a second generation *Himar1* construct employed the CBU0311 (outer membrane porin P1) promoter (*311*^{P}) to drive expression of mCherry independent of *1169*^{P}-driven CAT (**Figure 2A**). CBU0311 is constitutively expressed at high levels during the *C. burnetii* infectious cycle (P. A. Beare and R. A. Heinzen, unpublished data). A cloned transformant termed MC1 with an intergenic *Himar1* insertion between CBU0316 and CBU0317 was obtained that shows no growth defect in Vero cells relative to wild-type *C. burnetii* (Beare et al., 2010). By both fluorescence microscopy and flow cytometry, mCherry protein fluorescence was significantly higher in MC1 than the previously described B2c clone (Beare et al., 2009; **Figures 2B,C**).

Acidified citrate cysteine medium facilitated development of two new *C. burnetii* transformation systems: (1) a shuttle vector that is compatible with the endogenous plasmid of the organism, and (2) a Tn7 system for single-copy, site-specific chromosomal gene integration. Plasmids with an RSF1010 *ori* were recently shown to autonomously replicate in *C. burnetii* (Chen et al., 2010; Voth et al., 2011) with a copy number of roughly 3–6 (P. A. Beare and R. A. Heinzen, unpublished data). Chen et al. (2010) constructed the vector pKM230 by inserting a cassette encoding mCherry and CAT genes, under control of *C. burnetii groES* (CBU1719) and *com1* (CBU1910) promoters, respectively, into the *Legionella pneumophila* plasmid pJB908 (Sexton et al., 2004). Transformation with pKM230 yielded chloramphenicol-resistant, mCherry-fluorescing *C. burnetii* as assessed by growth in both ACCM and L929 cells. Subsequently, a second plasmid called pCBTEM was constructed that allows identification of *C. burnetii* secreted proteins that are putative Dot/Icm type IV secretion system (T4SS) substrates. The secretion assay relies on cytosolic delivery of BlaM C-terminally fused to a *C. burnetii* protein containing a T4SS translocation signal. Once secreted, the BlaM moiety of the protein chimera cleaves the β-lactam ring of a cell loaded fluorescent compound (CCF4/AM), resulting in blue cytosolic fluorescence (Campbell, 2004; de Felipe et al., 2008). The mCherry/CAT cassette of pKM230 was cloned into the BlaM fusion vector pXDC61 (de Felipe et al., 2008) where BlaM expression is under control of IPTG-inducible *tac*^{P}, to generate pCBTEM. Infection of THP-1 cells with pCBTEM transformants confirmed translocation of six suspected T4SS effector proteins by *C. burnetii*. In a similar study, Voth et al. (2011) used both BlaM and adenylate cyclase (CyaA) translocation assays to show that the cryptic QpH1 plasmid of *C. burnetii* encodes several T4SS substrates. Two *C. burnetii–E. coli* shuttle vectors, termed pJB-CAT and pJB-KAN were first derived by modifying the *L. pneumophila* plasmid pJB2581 (Bardill et al., 2005) to encode CAT and Kan genes, respectively, under control of *1169*^{P}. These plasmids were then modified to become T4SS reporter plasmids by adding *1169*^{P}-driven *blaM* or *cyaA* (**Figure 3A**). Like the BlaM assay, the CyaA assay is an enzymatic reporter assay that relies on cytosol delivery of CyaA C-terminally fused to a *C. burnetii* T4SS effector protein. Once in the cytosol, the CyaA moiety is activated by binding cytosolic calmodulin, resulting in elevated levels of cyclic AMP (Sory and Cornelis, 1994). Both assays revealed that CBUA0015 is one of six QpH1 plasmid-encoded proteins that are secreted (Voth et al., 2011; **Figure 3B**). A transformation frequency (transformants/protocol) of approximately 5×10^{-5} has since been established for *C. burnetii* using pJB-CAT or pJB-KAN and ACCM culture techniques (Omsland et al., 2011). These vectors have multiple applications including *in trans* complementation of mutants generated by Tn or other mutagenesis techniques, expression of dominant/negative proteins, and expression of epitope tagged proteins for intracellular trafficking studies.

Gene dosage effects that skew phenotypes are a potential concern with the plasmid copy number of pJB-CAT/KAN vectors. As discussed above, *Himar1* can be used for *in cis* complementation and single-copy expression of a heterologous protein (e.g., mCherry; Beare et al., 2010; Clark et al., 2011). However, the highly random nature of *Himar1* genomic integration necessitates mapping and phenotyping of clones to ensure an insertion event has not generated an unintended phenotype. An alternative system of single-copy chromosomal transgene expression was developed in *C. burnetii* that employs the transposon Tn7. The Tn7 system utilizes a transposase encoded by *tnsABCD* that recognize a specific 30 bp site termed *att*Tn7 that, in *Pseudomonas aeruginosa* and other Gram-negative bacteria, is located in the extreme 3′ end of *glmS* encoding glucosamine-6-phosphate synthetase (Choi et al., 2005). Transposition is orientation specific and occurs 36 bp downstream of *att*Tn7 in an intergenic region (Choi et al., 2005). Analysis of *C. burnetii glmS* (CBU1787) revealed 24 nucleotide identity with the *P. aeruginosa att*Tn7 site, suggesting this region comprises a functional Tn7 recognition site. To test this possibility, a two-plasmid Tn7 system was constructed (Choi et al., 2005).

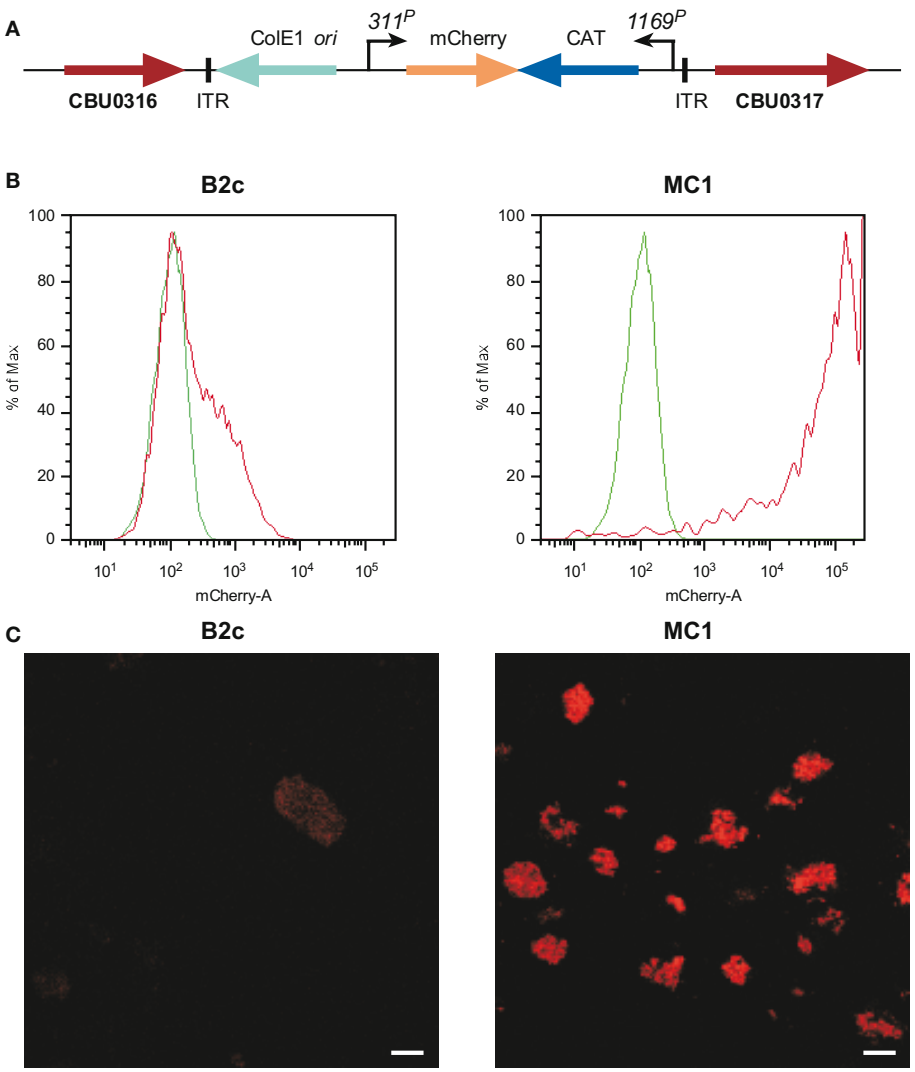

FIGURE 2 | Characterization *C. burnetii* Nine Mile (phase II) MC1 and B2c *Himar1* transformants. (A) Schematic of the *Himar1* chromosomal integration site in *C. burnetii* MC1. The *Himar1* transposon is flanked by inverted terminal repeat (ITR) elements and inserted into an intergenic region between CBU0316 and CBU0317. Flow cytometry **(B)** and confocal fluorescence microscopy **(C)** of live Vero cells infected with MC1 or B2c for 5 days. Both assays revealed considerably more mCherry fluorescence from MC1-containing vacuoles, where expression is driven by *311*^{P}, then from B2c-containing vacuoles, where expression is driven by *1169*^{P} (Beare et al., 2009). The green trace in flow cytometry histograms shows autofluorescence of Vero cells infected with wild-type *C. burnetii* for 5 days. Bars, 5 μm.

One suicide plasmid, termed pTNS2::*1169*^{P}*-tnsABCD*, carries the transposase genes *tnsABCD* under control of *1169*^{P}. The second suicide plasmid, called pMiniTn7T-CAT::GFP, carries Tn7 containing CAT and GFP genes driven independently by *1169*^{P} and *311*^{P}, respectively (**Figure 4A**). Co-electroporation of *C. burnetii* with pTNS2::1169^{P}*-tnsABCD* and pMiniTn7T-CAT::GFP, followed by chloramphenicol selection of transformants in ACCM, yielded clones with Tn7 inserted 36 bp downstream of the *att*Tn7 site in an intergenic region between *glmS* (CBU1787) and the hypothetical protein encoding gene CBU1788 (**Figure 4B**). Vero cells infected with expanded *C. burnetii* Tn7 transformants display high levels of GFP expression when visualized by fluorescence microscopy at 5 days post-infection (**Figure 4C**).

CHLAMYDIA TRACHOMATIS AND *CHLAMYDIA PSITTACI*

A perhaps underappreciated fact is that Tam et al. (1994) published the first description of genetic transformation, albeit transient in nature, of an obligate intracellular bacterium in 1994. *C. trachomatis* was transformed to chloramphenicol resistance by electroporation with the plasmid pPBW100. The plasmid contains 3.6 kb of an *E. coli* ColE1 *ori* cloning vector and 7 kb of pCTE1, the 7.5-kb endogenous plasmid of *C. trachomatis* serovar E. For antibiotic selection, a CAT gene was also cloned immediately downstream from a native chlamydial promoter. Electroporated EB, the infectious developmental form of chlamydiae, were used to infect McCoy cells and inclusions containing chloramphenicol-resistant chlamydia were initially detected. However, these were

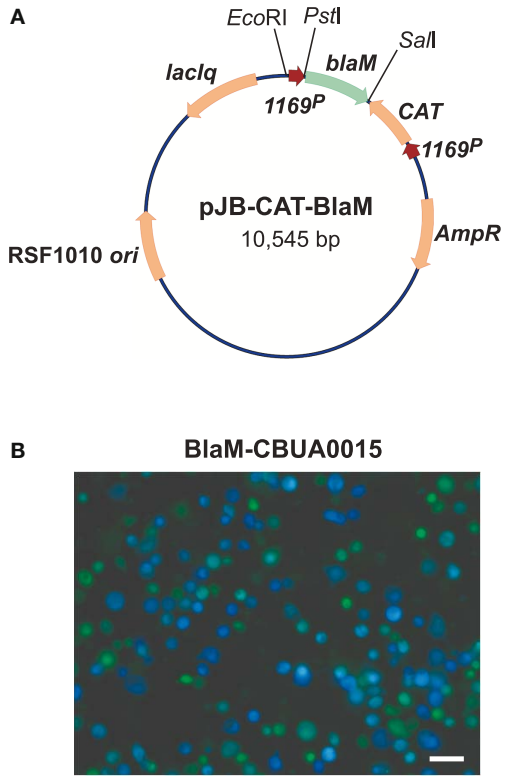

FIGURE 3 | A *C. burnetii* BlaM translocation assay shuttle vector. (A) Map of the reporter plasmid pJB-CAT-BlaM which contains an RSF1010 *ori* that functions in both *E. coli* and *C. burnetii*. Genes encoding suspected secreted proteins are cloned downstream and in-frame with *blaM* using the unique SalI site. **(B)** BlaM translocation assay showing cytosolic delivery of a BlaM-CBUA0015 fusion protein. THP-1 cells were infected with *C. burnetii* Nine Mile (phase II) containing pJB-CAT-BlaM::CBUA0015 for 48 h, then incubated for 1 h with CCF4/AM. Cleavage of CCF4/AM by cytosolic BlaM results in blue fluorescent cells and indicates secretion of the fusion protein. Bar, 30 μm.

lost following several tissue culture passes. Suggested reasons for the transient nature of transformants included incompatibility of pPBW100 with pCTE1, activation of DNA restriction/modification systems that degraded pPBW100, and developmental regulation of the promoter driving CAT gene expression that resulted in insufficient levels of expression during the chlamydial developmental cycle (Tam et al., 1994). Nonetheless, this study was a technological triumph by showing that DNA could be introduced into metabolically inert EB by electroporation, that expression of heterologous selectable markers could be achieved, and that transformants could be detected in cell culture.

Fifteen years elapsed before the next successful transformation of *Chlamydia* spp. *C. psittaci* was transformed by allelic exchange using both linear and circular DNA containing an allele of the 16s rRNA gene of the organism engineered to contain mutations conferring resistance to kasugamycin and spectinomycin (Binet and Maurelli, 2009). EB were electroporated with different amounts of DNA that contained up to 8.1 kb of the *C. psittaci* rRNA chromosomal region plus the 16s rRNA mutations. Molecular typing of antibiotic resistant plaques picked from L929 mouse fibroblast monolayers revealed that transformation had occurred by homologous recombination, mediated by a minimum of two crossover events, with both linear and circular DNA substrates. The highest transformation frequencies (approximately 3×10^{-6}) were observed using 10–20 μg of circular DNA propagated in a methylase-deficient *E. coli* strain. Recombination frequencies using plasmid templates dropped almost 10-fold when rRNA homologous DNA was reduced from 8.1 to 2.5 kb. Similar to the situation with the rifampin resistance allele used in rickettsial transformation (Wood and Azad, 2000), kasugamycin, and spectinomycin resistance are recessive in a merodiploid strain. Thus, their use as selectable markers necessitates elimination of the wild-type gene, thereby limiting their utility in chlamydial transformation. Nonetheless, this study provided considerable information on optimal electroporation conditions and the state of DNA substrates that favor homologous recombination.

Chlamydial natural competence?

A growing body of evidence from genomic data and *in vitro* experiments indicates that chlamydiae actively engage in horizontal gene transfer and that the process drives genomic diversification and acquisition of antibiotic resistance genes (DeMars et al., 2007; DeMars and Weinfurter, 2008; Suchland et al., 2009). These observations are surprising as the chlamydial genomes are largely syntenous and contain a limited number of strain-specific genes. Our understanding of natural genetic exchange in chlamydia is based on several studies. Comprehensive genetic linkage analyses of *C. trachomatis* clinical isolates and genome wide comparative studies identified possible recombination hot spots flanking highly polymorphic loci (Gomes et al., 2007). OmpA and the Pmp family of proteins are surface exposed in chlamydiae and are among the most polymorphic gene products in this system (Lampe et al., 1993; Millman et al., 2001; Gomes et al., 2007; Nunes et al., 2010). The genetic variability of these genes suggests that recombination drives functional and/or antigenic variation that might be important for immune evasion. Individual patients are commonly infected with multiple strains; thus, it is logical to suspect that co-infections may provide the necessary reservoir of genetic material to drive intraspecies recombination and genome diversification (Suchland et al., 2003).

An artificial system was recently developed to study *in vitro* lateral gene transfer and recombination in chlamydia (DeMars et al., 2007; DeMars and Weinfurter, 2008; Suchland et al., 2009). Transfer of tetracycline, rifampin, ofloxacin, and/or clindamycin resistance is routinely detected between *C. trachomatis, Chlamydia suis*, or *Chlamydia muridarum* strains following coinfection of cells with differently resistant parental strains and selection for doubly resistant progeny. A dynamic range of DNA fragment sizes incorporate into recipient strains ranging from 100 bp up to 790 kbp (DeMars and Weinfurter, 2008; B. M. Jeffrey and D. D. Rockey, unpublished data) with recombination occurring throughout the genome without apparent site specificity. Each of the three *Chlamydia* species mentioned above occupy and grow within the same vacuole, but whether or not co-occupancy of an inclusion is a requirement for horizontal gene transfer in chlamydia is unknown.

It is difficult to determine how chlamydial DNA exchange occurs in the absence of identifiable conjugative machinery, competence inducing genes or pathways, restriction modification systems, or

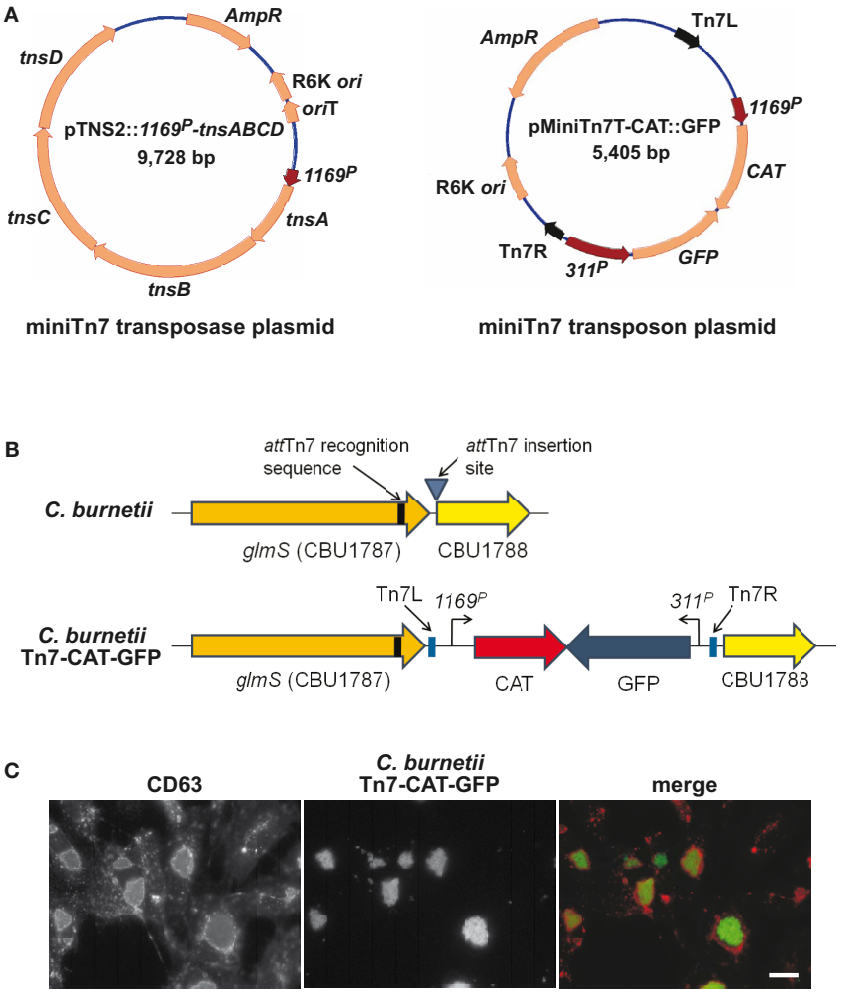

FIGURE 4 | *Coxiella burnetii*Tn7 transformation system. (A) Maps of two-plasmid *C. burnetii* miniTn7 transposon system. The suicide plasmid pTNS2*::1169*^{P}*-tnsABCD* encodes the *tnsABCD* operon under control of *1169*^{P}. The suicide plasmid pMiniTn7T-CAT::GFP encodes CAT and GFP genes driven independently by *1169*^{P} and *311*^{P}, respectively. The CAT/GFP cassette is flanked by Tn7L and Tn7R elements. **(B)** Schematic of the *glmS* regions in *C. burnetii* Nine Mile (phase II) and *C. burnetii* Nine Mile (phase II)/Tn7-CAT-GFP. **(C)** Fluorescence microscopy of Vero cells infected for 5 days with *C. burnetii* Nine Mile (phase II)/Tn7-CAT-GFP (green). Cells were fixed with 4% paraformaldehyde, then immunostained for the lysosomal protein CD63 (red). Bar, 5 μm.

marked genomic differences among strains. However, it is possible that DNA is transferred between chlamydiae via uptake of extracellular DNA released by lysed organisms. If so, perhaps this "natural competence" could be exploited to develop a transformation system for chlamydiae, an exciting possibility for these organisms that are still genetically intractable relative to other obligate intracellular bacteria.

A reverse genetic approach to genetic manipulation of C. trachomatis

An exciting new development in genetic manipulation of *Chlamydia* spp. that circumvents difficulties in genetic transformation was recently described by Kari et al. (2011) who used a reverse genetic approach to generate isogenic mutants of *C. trachomatis*. The strategy is based on an approach frequently used in plant genetics called Targeting Induced Local Lesions in Genomes (TILLING; McCallum et al., 2000). *C. trachomatis* was first chemically mutagenized with ethyl methanesulfonate in a manner that generated an estimated 0.5 mutations per genome. Mutagenized chlamydiae in pools of roughly 10 organisms were then expanded in McCoy cells in individual wells of 96 well plates. Genomic DNA was isolated from a duplicate library for use in PCR. To provide proof of principle, a PCR-based screen of pool DNA was conducted to detect mutations in *trpB*, encoding tryptophane synthase beta chain. PCR amplicons derived from pools were digested with the mismatch specific endonuclease CEL I. Amplicons containing mismatch mutations as detected by agarose gel electrophoresis were nucleotide sequenced to identify single nucleotide polymorphisms (SNPs). Of the 2,800 pools screened, 13 contained non-synonymous SNPs in *trpB*, with one being a nonsense mutation predicted to disrupt TrpB function. Unlike wild-type bacteria, the plaque-cloned mutant could not be rescued with indole during interferon-γ induced tryptophane depletion. While labor intensive, this approach represents the only technology currently available for generating isogenic null mutants of chlamydiae. As mentioned

above, and in keeping with other obligate intracellular bacteria, null mutations in genes required for growth in cell culture will not be attainable using this method. However, knockouts in genes that function primarily in animal infection and pathogenesis, such as TrpB, implicated in strain-specific tissue tropism (Caldwell et al., 2003), can be accomplished.

PERSPECTIVE

Successful transformation of several obligate intracellular bacteria proves that barriers to genetic manipulation, while often significant, are not insurmountable. Electroporation is universally effective in introducing heterologous DNA into these bacteria. Positive selection of transformants using resistance to antibiotics is feasible despite restricted use of antibiotics based on clinical efficacy. Homologous recombination and transposition occur at some frequency.

While genetic methods for obligates are still rudimentary compared to those available for most free-living bacterial pathogens, several milestones have been reached. The *Himar1* transposon has proven particularly useful with *Coxiella*, *Rickettsia*, and *Anaplasma* in generating transformants expressing fluorescent proteins with applications in pathogen–host interaction studies. *Himar1*-mediated gene inactivation also revealed a novel virulence gene (*sca2*) in *R. rickettsii* and, by *in cis* complementation of a naturally occurring mutant, identified a *relA/spoT*-like gene as involved in the lytic plaque phenotype of the organism. The first targeted gene disruption using transformation in any obligate intracellular bacterium was achieved with the knockout of *pld* in *R. prowazekii*. Recent axenic growth of *C. burnetii* is significantly aiding development of genetic tools that now include *Himar1* Tn mutagenesis, RSF1010 *ori*-based shuttle vectors, and a Tn7 system for single-copy, site-specific gene delivery. Finally, progress has been made in understanding requirements for transformation of the most recalcitrant of obligates, *Chlamydia* spp. A chronological overview of achievements in genetic transformation of obligate intracellular bacterial pathogens is detailed in **Table 1**.

Notwithstanding these notable achievements, additional advances in genetic manipulation of obligates are still required before molecular dissection of virulence factors can become routine. First and foremost, efficient methods of targeted gene disruption and complementation are needed. Traditional methods of allelic exchange rely upon double crossover events between a mutated allele of a gene, carried by a suicide plasmid or linear fragment, and the wild-type copy on the chromosome. The low frequency of double crossover events, combined with the poor transformation efficiencies of obligates (discussed in more detail below), makes this approach difficult. Counterselectable markers, such as *sacB* conferring sucrose sensitivity, can dramatically aid recovery of gene knockout mutants (Reyrat et al., 1998). In this strategy, chromosomal integration of a suicide plasmid by a single crossover event between a plasmid-coded mutant allele and the chromosomal wild-type gene is first achieved by positive selection for antibiotic resistance. A second crossover event that resolves the co-integrant and generates the desired mutation is then selected for by growth under counterselective conditions (e.g., media containing sucrose). While widely used in free-living bacteria to generate both marked and unmarked mutants, novel counterselective strategies may be necessary for obligate intracellular bacteria. Another possibility for targeted gene disruption in obligates involves mobile group II introns (targetrons). This technique is effective in other fastidious bacteria (Rodriguez et al., 2008) and relies on retargeting of the intron to a gene of interest by a process referred to as "retrohoming" (Karberg et al., 2001). Finally, recombineering using bacteriophage λ Red recombination functions should be considered (Thomason et al., 2007). λ Red functions promote high efficiency *in vivo* homologous recombination of sequences with homologies as short as 40 bases. Site-directed gene disruption might be accomplished by co-transforming with a linear targeting sequence and a suicide plasmid expressing of the λ Red recombination enzymes. With the exception of *C. burnetii*, plasmid shuttle vectors are needed for *in trans* complementation and gene expression studies using reporters such as *lacZ* and destabilized forms of GFP (Barysheva et al., 2008). Systems for inducible gene expression are also desirable for understanding protein function. For example, gene induction during a specific stage of the infectious cycle can be useful for determining the temporal requirement of a specific virulence factor.

Although robust gene knockout technologies are clearly needed for obligate intracellular bacteria, any gene inactivation that lowers fitness for intracellular growth may preclude isolation of the desired mutant. Indeed, to date, just a handful of genes have been inactivated in obligate intracellular bacteria. Isolation of null mutants of obligate intracellular bacteria may be particularly problematic due to their extensively downsized genomes. Nonetheless, there are clearly a subset of genes that are not essential for growth in cultured cells but play important roles in animal colonization and virulence. Rickettsial *pld* and *sca2* (Driskell et al., 2009; Kleba et al., 2010), and chlamydial *trpB* (Kari et al., 2011), are examples. A system for inducible *in trans* expression may allow inactivation of genes essential for intracellular growth (Molofsky and Swanson, 2003). These procedures typically involve creation of a strain carrying a plasmid with an inducible copy of a chromosomal gene targeted for inactivation. IPTG induction of *lacI-tac*^{p} works with intracellular *Legionella pneumophila* (Roy et al., 1998) and may be similarly effective with obligates.

Low transformation efficiencies remain an obstacle to further development of genetic tools for obligates. Many reasons could account for poor efficiency including suboptimal electroporation conditions/buffers, purity of host cell-derived organisms, and restriction/modification systems that degrade heterologous DNA. Improvements in transformation efficiencies that allow near saturation-level Tn mutagenesis might permit identification of genes required for host cell/animal infection by deep sequencing technologies (Gawronski et al., 2009). Cloning also remains a challenge for non-plaque-forming organisms, but limiting dilution and micromanipulation are alternatives (Suhan et al., 1996; Rachek et al., 2000; Qin et al., 2004; Beare et al., 2007).

The lack of methods to genetically manipulate obligate intracellular bacteria has historically impeded progress in understanding the genetic basis of their unique lifestyles and virulence. However, recent achievements in genetic transformation of nearly all medically relevant genera of this group will undoubtedly fuel new interest in these understudied but fascinating

Table 1 | History of genetic transformation of obligate intracellular bacterial pathogens.

Genus	Species	Transformation achievement	Reference
Anaplasma	*phagocytophilum*	*Himar1*-mediated random transposition resulting in spectinomycin-resistant, GFP-expressing bacteria	Felsheim et al. (2006)
	marginale	Creation of GFP-expressing bacteria via homologous recombination between the *tr* promoter on a suicide plasmid containing GFP and the *tr* region on the chromosome	Felsheim et al. (2010)
Chlamydia	*trachomatis*	Transient expression of chloramphenicol resistance by transformation with a potential shuttle vector comprised of the endogenous chlamydial plasmid and an *E. coli* cloning vector	Tam et al. (1994)
	psittaci	Allelic exchange between the chromosomal 16s rRNA gene and an allele containing mutations conferring resistance to kasugamycin and spectinomycin carried by either linear DNA or a suicide plasmid	Binet and Maurelli (2009)
Coxiella	*burnetii*	Transformation to ampicillin resistance via homologous recombination between pSKO(+)1000 and the chromosome, or autonomous replication of pSKO(+)1000	Suhan et al. (1996)
	burnetii	Generation of bacteria that weakly express GFP by transformation with pSKO(+)1000 containing *gfp*	Lukacova et al. (1999)
	burnetii	*Himar1*-mediated random transposition resulting in chloramphenicol-resistant, mCherry-expressing bacteria. Characterization of an *ftsZ Himar1* mutant.	Beare et al. (2009)
	burnetii	Development of RSF1010 *ori*-based shuttle vector. Use of shuttle vector in β-lactamase-based secretion assay.	Chen et al. (2010)
	burnetii	Development of RSF1010 *ori*-based shuttle vector. Use of shuttle vector in β-lactamase- and adenylate cyclase-based secretion assays.	Voth et al. (2011)
	burnetii	Optimization of the *Himar1* system for increased mCherry fluorescence	This study
	burnetii	Development of a miniTn7 transposon system for single-copy, site-specific transposon integration into the chromosome	This study
Rickettsia	*prowazekii*	Transformation to rifampin resistance via homologous recombination between a mutant *rpoB* gene on a suicide vector and the wild-type gene on the chromosome	Rachek et al. (1998)
	typhi	Creation of GFP-expressing bacteria via homologous recombination between wild-type *rpoB* on a suicide plasmid containing *gfp* and *rpoB* on the chromosome	Troyer et al. (1999)
	prowazekii	Transformation to erythromycin resistance via homologous recombination between wild-type *gltA* on a suicide vector containing *ereB* and *gltA* on the chromosome	Rachek et al. (2000)
	conorii	Creation of GFP-expressing bacteria via homologous recombination between wild-type *rpoB* on a suicide plasmid containing *gfp* and *rpoB* on the chromosome	Renesto et al. (2002)
	prowazekii	EZ::Tn5-mediated random transposition resulting in rifampin-resistant rickettsia	Qin et al. (2004)
	monacensis	EZ::Tn5-mediated random transposition resulting in chloramphenicol-resistant, GFP-expressing rickettsia	Baldridge et al. (2005)
	prowazekii	*Himar1*-mediated random transposition resulting in rifampin-resistant, GFP-expressing rickettsia	Liu et al. (2007)
	prowazekii	Inactivation of the phospholipase D gene (*pld*) by allelic exchange	Driskell et al. (2009)
	montanensis	EZ::Tn5-mediated random transposition resulting in chloramphenicol-resistant, GFP-expressing rickettsia	Baldridge et al. (2010b)
	rickettsii	Insertional inactivation of *sca2* by *Himar1* resulting in rifampin-resistant rickettsia lacking actin-based motility	Kleba et al. (2010)
	rickettsii	Creation of non-lytic rickettsia by *in cis* expression of *relA*/*spoT* carried by *Himar1*	Clark et al. (2011)

bacterial pathogens. Greater genetic tractability is inevitable, and will lead to novel insight into intracellular parasitism and disease pathogenesis, in addition to enabling development of new pathogen countermeasures, such as rationally designed attenuated or subunit vaccines.

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Adherence to and invasion of host cells by spotted fever group *Rickettsia* species

Yvonne Gar-Yun Chan, Sean Phillip Riley and Juan Jose Martinez*

Department of Microbiology, University of Chicago, Chicago, IL, USA

Edited by:
Robert Heinzen, National Institute of Health, National Institute of Allergy and Infectious Diseases-Rocky Mountain Laboratories, USA

Reviewed by:
Jon Audia, University of South Alabama School of Medicine, USA
Hélène Marquis, Cornell University, USA

***Correspondence:**
Juan Jose Martinez, Department of Microbiology, Cummings Life Sciences Center, University of Chicago, 920 E. 58th Street, CLSC701A, Chicago, IL 60637, USA.
e-mail: jmartine@bsd.uchicago.edu

The pathogenic lifecycle of obligate intracellular bacteria presents a superb opportunity to develop understanding of the interaction between the bacteria and host under the pretext that disruption of these processes will likely lead to death of the pathogen and prevention of associated disease. Species of the genus *Rickettsia* contain some of the most hazardous of the obligate intracellular bacteria, including *Rickettsia rickettsii* and *R. conorii* the causative agents of Rocky Mountain and Mediterranean spotted fevers, respectively. Spotted fever group *Rickettsia* species commonly invade and thrive within cells of the host circulatory system whereby the endothelial cells are severely perturbed. The subsequent disruption of circulatory continuity results in much of the severe morbidity and mortality associated with these diseases, including macropapular dermal rash, interstitial pneumonia, acute renal failure, pulmonary edema, and other multisystem manifestations. This review describes current knowledge of the essential pathogenic processes of adherence to and invasion of host cells, efforts to disrupt these processes, and potential for disease prevention through vaccination with recently identified bacterial adherence and invasion proteins. A more complete understanding of these bacterial proteins will provide an opportunity for prevention and treatment of spotted fever group *Rickettsia* infections.

Keywords: *Rickettsia*, adherence, invasion

INTRODUCTION

The Gram-negative α-proteobacteria of the genus *Rickettsia* are small (0.3–0.5 × 0.8–1.0 μm), obligate intracellular organisms. They are categorized into two major groups, the spotted fever group (SFG) and typhus group (TG), which can be distinguished by antigenicity and intracellular actin-based motility. Members of this genus are responsible for severe human diseases and several species including *Rickettsia rickettsii*, *R. prowazekii*, and *R. typhi*, have been classified as Category B and C Priority Pathogens by the National Institute of Allergy and Infectious Diseases (NIAID) and as Select Agents (*R. rickettsii* and *R. prowazekii*) by the Centers for Disease Control and Prevention (CDC) for their potential use as tools for biological terrorism.

Spotted fever group rickettsiae including *R. rickettsii* (Rocky Mountain spotted fever, RMSF) and *R. conorii* (Mediterranean spotted fever, MSF) are pathogenic organisms transmitted to humans through tick salivary contents during the blood meal. RMSF is one of the most severe SFG rickettsioses in the western hemisphere, causing severe morbidity and up to 20% mortality in the absence of timely and appropriate antibiotic treatment (Walker, 1989). MSF, endemic to southern Europe, North Africa and India, has previously been characterized as a milder rickettsiosis in humans, with 2–3% mortality; however, in light of improved molecular diagnostic tools, recent accumulating evidence has unveiled that MSF exhibits an expansive geographic distribution, now including central Europe and central and southern Africa, and increased disease severity commensurate to RMSF, with mortality rates reported as high as 32% in Portugal in 1997 (de Sousa et al., 2003). This raises concern over the challenges posed by *R. conorii* infection.

Symptoms from rickettsial disease manifest 2–14 days following inoculation by an infected ixodid tick. Early indications of infection are unremarkable and include headache, fever, and malaise. Soon after the tick bite, localized replication of rickettsiae at the inoculation site and ensuing tissue damage may give rise to a necrotic lesion, or eschar. Damage to the vascular endothelium and infiltration of perivascular mononuclear cells leads to fluid leakage into the interstitial space resulting in a dermal rash in 90% of cases. Endothelial cells are the main targets during rickettsial infection. Bacterial replication within the endothelial tissues and subsequent damage to the integrity of the vasculature leads to complications such as encephalitis, non-cardiogenic pulmonary edema, interstitial pneumonia, hypovolemia, hypotensive shock, and acute renal failure (Walker et al., 1994).

The TG rickettsiae include *R. prowazekii*, the etiologic agent of epidemic typhus, and *R. typhi*, the causative agent of murine typhus. Epidemic typhus outbreaks have been documented in disparate global communities (Bise and Coninx, 1997; Raoult et al., 1997, 1998), thriving in areas of poor sanitation and hygiene. TG rickettsiae are normally and stably transmitted through the excrement of human body lice and are inoculated into abraded skin by scratching. Early signs of infection are characterized by headaches, fever, delirium, and rash. Delayed or inappropriate treatment can result in mortality rates ranging from 10 to 60%. Unlike SFG rickettsial diseases, *R. prowazekii* can cause latent infections, where recurrence results in Brill–Zinsser disease, a less severe but chronic *R. prowazekii* infection that can be transmitted to feeding lice, thus fueling epidemics.

Rickettsia species are obligate intracellular bacteria and as such have evolved to take full advantage of the nutrient and energy-rich environment of the cytosol of host cells. In doing so, they have

undergone reductive evolution, discarding many of their own genes necessary for metabolite synthesis. Their succinct genome has made them completely dependent on the intracellular environment of the mammalian host cell for growth and survival. During an infection, rickettsial pathogenesis depends initially on the bacteria's ability to attach to and invade the host's cells. This requires successful recognition and interaction with specific cellular receptors, and is thought to be dependent on the presence of heat-labile proteins on the rickettsial surface (Li and Walker, 1992). While *Rickettsia* primarily infect the host endothelium, *in vitro* they are seen to adhere to and invade diverse types of mammalian cells (Cohn et al., 1959; Ramm and Winkler, 1973; Winkler, 1974, 1977; Winkler and Ramm, 1975; Stork and Wisseman, 1976; Wisseman et al., 1976; Walker and Winkler, 1978; Rikihisa and Ito, 1979; Turco and Winkler, 1982; Walker, 1984; Teysseire et al., 1995), in a mechanism requiring host membrane cholesterol (Ramm and Winkler, 1976; Martinez and Cossart, 2004). This review addresses current knowledge of SFG *Rickettsia* adherence to and invasion of host cells, with particular emphasis on the host signaling mechanisms induced by the bacteria, the bacterial proteins that mediate these processes, and efforts to utilize these rickettsial proteins in vaccination.

RICKETTSIA INVASION

Intracellular bacterial pathogens have been shown to facilitate their entry into non-phagocytic host cells by either of two mechanistically and morphologically distinct means: the "zipper" or "trigger" mechanisms (reviewed in Alonso and Garcia-del Portillo, 2004). The zipper invasion mechanism is a receptor-mediated invasion strategy, whereby a bacterial protein induces host intracellular signaling through extracellular stimulation of a membrane receptor. These signals modulate local host cytoskeletal rearrangements and recruitment of endocytic machinery at the site of interaction, culminating in membrane "zippering" around the pathogen (Cossart, 2004). Hallmark examples of this are seen in *Listeria monocytogenes* with InlA and InlB, and *Yersinia pseudotuberculosis* YadA and invasin (Mengaud et al., 1996; Wong and Isberg, 2003; Alonso and Garcia-del Portillo, 2004; Veiga and Cossart, 2005, 2007). In contrast, the "trigger" mechanism relies on Type III secretion system (TTSS)-mediated delivery of bacterial effectors into the host cell to modulate cellular GTPases and give rise to dramatic actin and membrane rearrangements (Alonso and Garcia-del Portillo, 2004). Genome analyses of sequenced strains of the genus *Rickettsia* show the absence of genes encoding for components of the TTSS, suggesting that rickettsial species likely induce their internalization into non-phagocytic mammalian cells by a mechanism independent of this secretion machinery. Interestingly, transmission electron micrographs of *R. conorii* entry illustrate that, like *L. monocytogenes*, rickettsial invasion is characterized by intimate localized rearrangement of cellular plasma membrane around the bacteria morphologically resembling a zipper-induced entry mechanism (Teysseire et al., 1995; Gouin et al., 1999).

Numerous detailed analyses of invasion into non-phagocytic mammalian cells have demonstrated that host actin polymerization plays a critical role during the induced entry of rickettsial species (Walker and Winkler, 1978; Walker, 1984; Gouin et al., 1999; Martinez and Cossart, 2004). An initial examination of proteins directly involved in modulating actin dynamics during *R. conorii* invasion illustrated the involvement of the small GTP-binding protein, Cdc42, and the actin nucleating complex, Arp2/3, in the invasion process (Martinez and Cossart, 2004). These proteins are required for rickettsial entry and localize to the sites of *R. conorii* invasion as revealed by immunofluorescence microscopy. Specific perturbation of these players, for example, inhibition of Arp2/3 function by transfection of the WASP family member, Scar, or Cdc42 through transfection of a dominant-negative variant, diminishes rickettsial invasion. The GTPase activity governing the actin cytoskeletal rearrangements was seen to be specific for Cdc42, as similar inhibition of Rac1 had no effect on the efficiency of *R. conorii* invasion. Pharmacological inhibition of pathways that both directly and indirectly modulate the activities of Cdc42 and Arp2/3 showed that actin polymerization, PI 3-kinases, phosphotyrosine kinase (PTK) activities and the activity of a specific PTK, c-Src, contribute to *R. conorii* invasion of non-phagocytic mammalian cells (Martinez and Cossart, 2004). Examination of c-Src and one of its downstream effectors, cortactin, by immunofluorescence microscopy showed that both are also recruited to sites of rickettsial internalization, highlighting the importance of these proteins to the entry process (Martinez and Cossart, 2004). In addition, *R. conorii* invasion coincides with the tyrosine phosphorylation of several host proteins including focal adhesion kinase (FAK) (Martinez and Cossart, 2004), a protein involved in cytoskeletal reorganization and in the invasion of other "zippering" pathogens (Persson et al., 1997; Martinez et al., 2000). Together, these findings indicate that signaling events from these various components are likely coordinated to ultimately activate Arp2/3, thus instigating localized actin recruitment around the invading bacteria. While these studies provide insight into the signaling events involved in stimulating *R. conorii* entry of non-phagocytic mammalian cells, until recently, little was known about the identity of the rickettsial proteins and cellular receptors and their interplay at the extracellular interfaces that govern the induction of these pathways.

IDENTIFICATION OF A *R. CONORII* HOST CELL RECEPTOR

In an effort to identify putative host cell receptors involved in rickettsial entry, whole, intact *R. conorii* were used as an affinity matrix to identify interacting mammalian host cell proteins (Martinez et al., 2005). This investigation led to the identification of host Ku70 as one of several cellular proteins that specifically interact with purified *R. conorii* and not other invasive pathogens. Ku70's role as a receptor for rickettsial entry was initially highlighted by immunofluorescence microscopy, which demonstrated co-localization of plasma membrane Ku70 with invading bacteria (Martinez et al., 2005). Further, pre-incubation of cells with antibody directed against a surface-exposed, extracellular epitope of Ku70 reduces *R. conorii* entry into mammalian cells, suggesting interaction of rickettsiae with this protein is important for stimulating bacterial internalization. An analysis of *R. conorii* invasion in cells either deficient ($Ku70^{-/-}$ murine embryonic fibroblasts) or siRNA-depleted of Ku70 reveal that invasion into these cells is perturbed at least 50% compared to controls (Martinez et al., 2005), indicating that the presence of cellular Ku70 is important for the rickettsial entry process. Interestingly, cells lacking Ku70 are not completely impervious to infection by *R. conorii*, implying that additional host proteins are likely utilized in rickettsial invasion (Martinez et al., 2005).

Ku70 is ubiquitously expressed in mammalian cells and is generally localized to the nucleus and cytoplasm (Muller et al., 2005). Expression of Ku70 at the plasma membrane is restricted to specific cell types including endothelial cells, monocytes and macrophages, which are the main target cells during rickettsial infection *in vivo*, and cultured tumor cell lines such as HeLa and Vero (Muller et al., 2005), both commonly utilized to culture *Rickettsia in vitro* (Martinez and Cossart, 2004; Ammerman et al., 2008). Because Ku70 lacks the canonical signal sequence that typically directs plasma membrane-destined polypeptides through the endoplasmic reticulum/golgi secretory pathway, its presentation in the extracellular environment was baffling. Recent studies examining induced Ku70 plasma membrane display in monocytes have shown that Ku70 is transported via a non-classical vesicle secretion mechanism (Paupert et al., 2007). Here, cytoplasmic Ku70 is gathered into membrane-bound vesicles and trafficked in the cytoplasm along the actin cytoskeleton for secretion and display at the plasma membrane. Biochemical fractionations of mammalian cells have shown that plasma membrane-associated Ku70 is present in cholesterol-rich, detergent-resistant microdomains or "lipid-rafts" (Martinez et al., 2005), and that depletion of cholesterol from HeLa or Vero cells by methyl-β-cyclodextrin disrupts the integrity of these microdomains and prevents rickettsial entry (Martinez et al., 2005). While these studies illustrate the presence of Ku70 at the plasma membrane, and its integral role in rickettsial invasion, the exact topology of the protein is unknown and is currently under investigation.

Attachment of invasive pathogens to host cell receptors commonly leads to receptor modification and subsequent endocytic signaling that enable bacterial entry (Su et al., 1999; Eto et al., 2007; Veiga and Cossart, 2007). Examination of the modification status of Ku70 consequent to rickettsial infection reveal that upon bacterial attachment, Ku70 is ubiquitinated by c-Cbl, an E3 ubiquitin ligase, which by fluorescence microscopy is seen to localize to *R. conorii* entry foci (Martinez et al., 2005). Inhibition of endogenous c-Cbl expression by siRNA depletion effectively reduces rickettsial invasion and also prevents *R. conorii*-induced ubiquitination of Ku70, suggesting that c-Cbl modification of Ku70 may contribute to *R. conorii* entry (Martinez et al., 2005). Mechanistically this process resembles *L. monocytogenes* InlB induction of c-Met receptor mono-ubiquitination, which is involved in subsequent clathrin-dependent endocytosis (Veiga and Cossart, 2005). The morphological and mechanistic similarities shared amongst "zippering" pathogens would suggest that ubiquitin-modification of bacterial receptors may be a generalized strategy to recruit components of the endocytic machinery to entry foci (see below).

THE RICKETTSIAL CELL SURFACE

Since rickettsial adherence and invasion are most likely mediated by components present on the surface of these bacteria, much research has focused on the identification and characterization of outer membrane-associated rickettsial proteins. Bioinformatic analyses of sequenced rickettsial genomes have identified families of proteins that likely localize to the outer membrane and are therefore predicted to be involved in mediating interactions with target host cells. Of interest was the identification of a family of at least 17 different genes termed *sca* for surface cell antigens whose products resemble autotransporter proteins, many of which are known virulence factors in Gram-negative bacteria (Henderson and Lam, 2001; Jacob-Dubuisson et al., 2004; Blanc et al., 2005). These proteins have modular structures, including an N-terminal signal peptide, a central passenger domain, and a C-terminal translocation module (β-peptide). Following translation, these proteins are secreted across the inner membrane using information encoded in the N-terminal signal sequence. The C-terminal domain then inserts into the outer membrane to form a β-barrel-rich transmembrane pore believed to serve in the secretion of the passenger domain into the extracellular milieu (Jacob-Dubuisson et al., 2004; **Figure 1**). While a significant amount of degradation of genes in the *sca* family has occurred in many phylogenetically related and divergent rickettsial species, five genes, namely *sca0* (*rompA*), *sca1*, *sca2*, *sca4*, and *sca5* (*rompB*) are highly conserved among the majority of SFG rickettsiae (Roux and Raoult, 2000; Blanc et al., 2005); the evolutionary maintenance of these genes suggests they may exhibit a functional role in rickettsial pathogenesis. Thus, a great deal of emphasis has been put forth to determine the contribution of these gene products in rickettsial host cell interactions.

RICKETTSIA SURFACE CELL ANTIGENS

rOMPB (Sca5)

rOmpB is evolutionarily conserved amongst all known rickettsial species, and is present in both SFG and TG rickettsia. Originally referred to as SpaP, SPA, or p120, its persistent and current nomenclature is rOmpB protein encoded by the *rompB* gene.

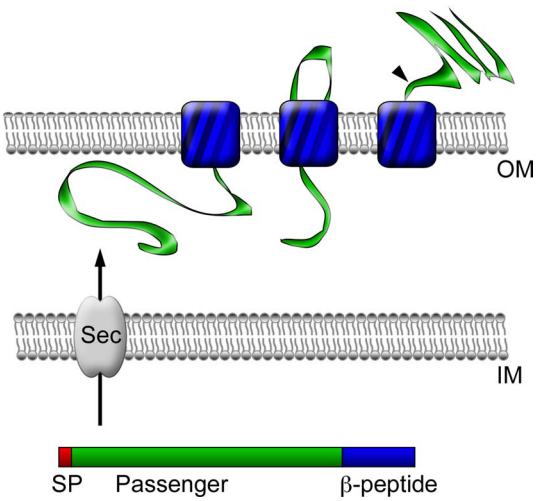

FIGURE 1 | Autotransporter protein structure and secretion. Autotransporter proteins have modular structures, including an N-terminal signal peptide (SP, red), a central passenger domain (green), and a C-terminal translocation module called a β-peptide (blue). Following translation, the polypeptide is secreted across the inner membrane (IM) through the Sec translocon into the periplasmic space using information encoded in the N-terminal signal sequence. Through an undefined mechanism, the β-peptide next inserts into the bacterial outer membrane (OM) to form a β-barrel-rich transmembrane pore for the secretion of the central passenger domain into the extracellular space. In the case of some proteins, such as rOmpB, the passenger domain is subsequently proteolyzed from the β-peptide portion of the protein, but may remain associated with the outer leaflet of the bacterial outer membrane.

Electron microscopic analyses of rickettsiae have revealed the presence of a 7–16 nm monolayer of protein arranged in a tetragonal array attached externally to the outer membrane (Palmer et al., 1974a,b; Popov and Ignatovich, 1976; Silverman and Wisseman, 1978; Silverman et al., 1978). This proteinaceous layer consists of 10–15% of total cellular protein and is composed of a diverse set of proteins, most predominantly rOmpB (Smith and Winkler, 1979; Dasch, 1981; Dasch et al., 1981; McDonald et al., 1988; Hechemy et al., 1989). Sequence analyses of rOmpB reveals a high level of conservation amongst diverse groups of rickettsiae (Blanc et al., 2005), exhibiting 70–95% identity on the amino acid level (**Table 1**). This conservation is evidence of positive selection which is apparent not only in the β-barrel domain which is restricted by its known function, but also proceeds into the predicted passenger domain (**Table 1**). These areas of conservation suggest rOmpB serves a unified function in rickettsial pathogenesis. rOmpB is expressed as a pre-protein (168 kDa) and cleaved to release the passenger domain (120 kDa) from the β-barrel translocation domain (32 kDa), leaving the mature 120-kDa domain associated with the outer leaflet of the outer membrane (Hackstadt et al., 1992). Interestingly, through affinity chromatography methods, Martinez et al. (2005) identified rOmpB as a bacterial ligand of the host receptor Ku70 suggesting that rOmpB–Ku70 serves as a *bona fide* adhesin-receptor pair in rickettsia-host cell interactions.

Recently, the roles of rOmpB in rickettsial pathogenesis, with regard to its contribution to bacterial adherence to and invasion of host cells, have been examined. Investigation of rOmpB function from related SFG rickettsiae, *R. conorii* and *R. japonica*, through heterologous expression of these proteins in *Escherichia coli* have shown that rOmpB is an integral player in initiating bacterial infection of mammalian host cells. When expressed on normally inert *E. coli*, rOmpB is sufficient to facilitate bacterial adherence to and invasion of non-phagocytic cells (Uchiyama et al., 2006; Chan et al., 2009; **Figure 2**). A previous study examining *R. conorii* entry showed that host protein, Ku70, served as a receptor for rickettsial invasion and interacted with rOmpB (Martinez et al., 2005). Bacterial invasion mediated by rOmpB alleles from both *R. conorii* and *R. japonica* were demonstrated to be Ku70-dependent (Chan et al., 2009), suggesting not only that rOmpB is a rickettsial adhesion and invasion molecule, but that it is the cognate bacterial ligand for the host receptor Ku70. Mammalian cells depleted of Ku70 no longer support rOmpB-mediated *E. coli* invasion, indicating that interaction of rOmpB and Ku70 are requisite to stimulate signals for bacterial uptake (Chan et al., 2009).

Mechanistic interrogation of the rOmpB–Ku70-mediated invasion process revealed that the interaction of these two proteins is sufficient to recruit the actin cytoskeleton to the bacterial entry foci on the cell (Chan et al., 2009). This rOmpB-mediated process, resembling that observed during *R. conorii* invasion, is manifested through the activities of PTKs and PI 3-kinases, and dependent on the cellular microtubule infrastructure (Martinez and Cossart, 2004; Chan et al., 2009). Similar to findings by Martinez et al., and paralleling the entry mechanisms observed in *R. conorii* infection, rOmpB-induced invasion via Ku70 is dependent on c-Cbl ubiquitin ligase activity, suggesting c-Cbl may be a signaling molecule involved in commandeering host endocytic pathways (Martinez and Cossart, 2004; Veiga and Cossart, 2005; Chan et al., 2009). Whether rOmpB is sufficient to mediate c-Cbl-dependent Ku70 ubiquitination during the entry process is not known. However, siRNA depletion of cellular players of endocytosis revealed that invasion through rOmpB is in part dependent on clathrin and caveolin-2 expression (Chan et al., 2009).

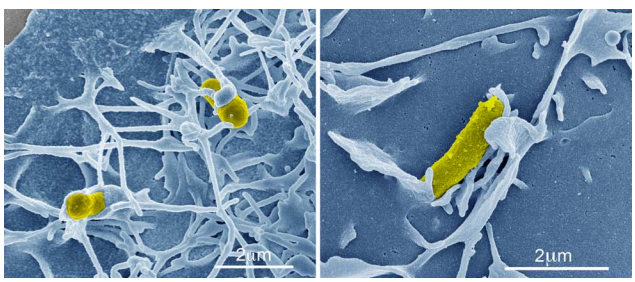

FIGURE 2 | rOmpB-mediated bacterial invasion. Pseudo-colored scanning electron micrographs of a rOmpB-expressing *Escherichia coli* (yellow) inducing host membrane rearrangements during infection of a mammalian HeLa cell (blue).

Table 1 | rOmpB sequence identity amongst SFG *Rickettsia* and *R. prowazekii*.

Species	Protein name	Predicted molecular weight (Da)	Protein identity (%)[1]	Passenger domain identity (%)[1,2]	β-Peptide identity (%)[1,2]
R. conorii Malish7	Rc1085	168,342	–	–	–
R. rickettsii Sheila Smith	A1G_06030	168,172	93.6	92.5	98.7
R. massiliae MTU5	RMA_1118	166,873	90.5	89.3	95.7
R. japonica YM	AAF34116.1	167,938	90.9	89.4	97.0
R. africae ESF5	YP_002845564.1	168,157	96.6	95.9	99.7
R. australis Phillips	AAF34112.1	167,484	80.1	77.5	89.0
R. prowazekii MadridE	RP704	169,841	71.4	69.3	80.6

[1]*Identity percentages based on R. conorii Malish7 rOmpB as determined by amino acid alignment performed in MacVector with ClustalW function and a gap penalty of 10.0.*

[2]*Amino acids conferring the* β*-peptide region were determined based on homology to protein family (PF) 03797.*

The specificity and physiological significance of rOmpB in its role in bacterial entry is underscored by the ability for recombinant rOmpB to competitively impede not only rOmpB-mediated *E. coli* association (Chan et al., 2009), but also *R. conorii* association with host cells (Y. G. Chan, unpublished data). The perturbation of this infection process can be elicited by the purified passenger domain (36–1334 aa), which additionally interacts directly with Ku70 (Chan et al., 2009). Although a previous study has proposed a putative role of the rOmpB β-peptide in adhesion (Renesto et al., 2006), these findings show that an effector region of this protein is in part contained within the passenger domain (Martinez et al., 2005; Chan et al., 2009).

rOmpA (Sca0)

The *rompA* gene is found in several SFG rickettsial species but is absent in TG *Rickettsia* (Blanc et al., 2005). In *R. rickettsii* strain Sheila Smith, *rompA* encodes for a protein of 247 kDa, while in *R. conorii* Malish 7 *rompA* encodes for a protein of 224 kDa. The final predicted lengths of rOmpA alleles vary from species to species, as is apparent in the disparate molecular weights of this protein between rickettsial strains. These differences are largely the result of variances in the number of tandem repeat sequences present within the passenger domain region of the protein (Eremeeva et al., 2003; Ellison et al., 2008). While nothing is known about the function of these repeat regions, their presence and conservation in a diverse set of rickettsial species suggests a conserved function in this protein (**Table 2**).

In comparison to rOmpB, very little is known about the putative processing of rOmpA in rickettsial species. The predicted rOmpA β-peptides of various *Rickettsia* species are nearly identical (**Table 2**). While bioinformatic models predict that the rOmpA signal sequence from *R. conorii* is demarcated by amino acids 1–38 (Emanuelsson et al., 2007), the identity of putative cleavage sites to release the passenger domain from the β-peptide is not known. Western immunoblot analyses of *R. conorii* detergent soluble lysates with rOmpA antisera have illuminated a reactive species of approximately 190 kDa, approximately 34 kDa less than the predicted mass of the full-length protein (Hillman and Martinez, unpublished observations) and (Vishwanath, 1991); similar results were observed in the mobility of the *R. rickettsii* rOmpA protein (Anderson et al., 1990). The molecular weight differences between the predicted and observed rOmpA might be due to aberrant mobility of the protein on SDS–PAGE; alternatively, these data suggest the intriguing possibility that rOmpA is proteolytically cleaved to release the passenger domain from an approximately 32–34 kDa beta-peptide translocon domain in the pre-protein, as has been observed for other autotransporters.

rOmpA has been shown to mediate rickettsial adherence (Li and Walker, 1998). Previously, monoclonal antibodies to *R. rickettsii* rOmpA and $F(ab')_2$ fragments derived from these antibodies have been demonstrated to inhibit the adhesion of rickettsiae to L-929 cells (Li and Walker, 1998). rOmpA extracted from intact *R. rickettsii* was further shown to be sufficient in competitively inhibiting attachment of *R. rickettsii* to these cells (Li and Walker, 1998), implicating a role for rOmpA in rickettsial association to mammalian cells. Another analysis of rOmpA function demonstrated that like rOmpB, rOmpA from *R. conorii,* when expressed at the outer membrane of *E. coli*, is sufficient not only to mediate bacterial adherence to but also invasion of cultured mammalian cells (Hillman and Martinez, unpublished observations). Together, these results suggest a critical role for rOmpA in mediating SFG *Rickettsia*-host cell interactions.

Sca1

sca1 is present in nearly all sequenced *Rickettsia* genomes, with the exceptions of *R. prowazekii* and *R. canadensis,* where it is present as a pseudo-gene (Ngwamidiba et al., 2006). Like rOmpA and rOmpB, Sca1 possesses all of the hallmarks of a surface-exposed autotransporter (Blanc et al., 2005). While selective pressure has limited the diversity of particular regions of the N-terminal and the β-peptide portions, the overall size of Sca1 varies significantly, ranging from 594 to 1976 aa (Ngwamidiba et al., 2006; Riley et al., 2010). However, the conservation of specific domains of Sca1 has led to the hypothesis that it serves universal functions amongst SFG *Rickettsia.*

Riley et al. (2010) demonstrated that Sca1 is expressed and present on the surface of *R. conorii* grown in mammalian cell culture. Flow cytometric analysis and immunofluorescence microscopy using a polyclonal antibody generated against the N- terminal portion (29–327 aa) of Sca1 provided evidence for surface localization

Table 2 | rOmpA sequence identity amongst SFG *Rickettsia.*

Species	Protein name	Predicted molecular weight (Da)	Identity (%)[1]	Passenger domain identity (%)[1,2]	β-Peptide identity (%)[1,2]
R. conorii Malish7	Rc1273	203,329	–	–	–
R. rickettsii Sheila Smith	A1G_06990	224,371	82.2	79.7	98.0
R. massiliae MTU5	RMA_1296	196,976	81.4	78.9	95.3
R. siberica 246	ZP_00142612	175,675	78.8	75.3	98.7
R. africae ESF-5	YP_002845686	210,512	87.5	85.6	99.3
R. australis PHS	AF149108	208,910	50.1	43.9	86.1

[1]*Identity percentages based on R. conorii Malish7 rOmpA as determined by amino acid alignment performed in MacVector with ClustalW function and a gap penalty of 10.0.*

[2]*Amino acids conferring the* β*-peptide region were determined based on homology to protein family (PF) 03797.*

of this protein in *R. conorii*, demonstrating that indeed Sca1 is exposed to the extracellular milieu (Riley et al., 2010). Interestingly, immunoblot analysis of *R. conorii* lysates with specific anti-Sca1 antisera revealed that Sca1 in *R. conorii* appeared to be processed to an approximately 120 kDa mature protein from the predicted greater than 200 kDa polypeptide (Riley et al., 2010). This finding complicates the historical assumption that rOmpB is the only antigenic protein at 120 kDa, since both the mature forms of Sca1 and rOmpB migrate similarly by SDS–PAGE analyses.

The *sca1* gene from *R. conorii* was expressed in *E. coli* and was demonstrated to be present in the outer membrane of these bacteria. The presence of Sca1 in *E. coli* was sufficient to mediate attachment to but not invasion of a panel of host cells (Riley et al., 2010). Finally, pre-incubation with a recombinant fragment of the Sca1 protein blocked some rickettsial association with host cells (Riley et al., 2010). Together, these findings indicate that adherence and invasion are two distinct events that can be mediated through multiple rickettsial proteins. Examination of the relative contribution of the rickettsial Sca proteins to adherence, invasion, and other potential cellular processes will need to be examined further.

Sca2

Sca2 is conserved and present in most SFG *Rickettsia* and either split or absent in TG species (Blanc et al., 2005; Haglund et al., 2010). The gene encodes for an approximately 200–220 kDa protein that ranges from 80 to 95% identity among SFG species, exhibiting the greatest conservation in the β-peptide and signal sequence (Ngwamidiba et al., 2005; Cardwell and Martinez, 2009). To date nothing is known about its potential processing and gene regulation, although numerous studies have demonstrated the gene is expressed during infection of mammalian cells and the protein is presented at the rickettsial surface (Cardwell and Martinez, 2009; Haglund et al., 2010).

The contribution of Sca2 to bacterial entry was examined by heterologous expression in *E. coli* (Cardwell and Martinez, 2009). In this system, the *R. conorii* Sca2 protein sufficiently mediated bacterial association to and invasion of numerous non-phagocytic mammalian cell types, most notably primary human endothelial cell lines, which are major targets during rickettsial infection. Furthermore, competitive inhibition of both *E. coli* as well as *R. conorii* invasion mediated through Sca2 was achieved using the N-terminal half of the Sca2 passenger domain (34–794 aa), suggesting this portion of the protein may be involved in mediating interactions with the host cell surface.

While Cardwell et al. were able to demonstrate a role for Sca2 in *R. conorii* entry (Cardwell and Martinez, 2009), new evidence has emerged indicating this may not be its sole function during infection of mammalian cell culture. In a recent transposon mutagenesis study, a *R. rickettsii sca2* mutant was discovered and seen to exhibit a defect in actin-based motility (Kleba et al., 2010). Proteomic analyses of the N-terminal portion of the Sca2 passenger domain sequence elucidated motifs resembling those present in eukaryotic actin modulating proteins, including three actin-binding WASP homology 2 domains and a predicted secondary structure resembling the formin homology 2 domain, suggesting a role for Sca2 in producing the observed parallel actin bundles involved in SFG rickettsial intra- and inter-cellular motility (Gouin et al., 1999; Haglund et al., 2010; Kleba et al., 2010). Comprehensive analyses of *R. parkeri* Sca2 by Haglund et al. (2010) further endorse the function of this protein in actin-based motility, showing Sca2 localization to actin-polymerizing surfaces of the bacteria, and demonstrating its *in vitro* capacity to polymerize actin monomers, and elongate filaments in a profilin-dependent manner. Together, these findings suggest that in the face of the limited genome size, rickettsial proteins may serve multiple pathogenic roles. Whether the invasion and actin-polymerizing functions of *R. conorii* Sca2 are composed of two distinct domains or are contained within the same region of the polypeptide is under investigation.

PUTATIVE RICKETTSIAL ADHESINS

A biochemical approach whereby rickettsial proteins resolved by SDS–PAGE were examined for interaction with surface-biotinylated mammalian cell extracts has unveiled several potential rickettsial adhesins. In addition to the rOmpB β-peptide, rickettsial gene products of RC1281 from *R. conorii* and RP828, its homologue in *R. prowazekii*, were identified as putative interactors of cellular membrane proteins (Renesto et al., 2006). RC1281/RP828 is conserved and ubiquitously present in the genomes of *Rickettsia* species and is paralogous to the adjacent gene RC1282 in *R. conorii*, which is also similarly conserved and prevalent but was not identified in that screen. BLAST analyses of these genes show a low level of homology to other bacterial surface proteins, some which have adhesin and toxin functions. Balraj et al. (2009) have reported that antibodies generated against the recombinant proteins encoded by RC1281 and RC1282 reduce *R. conorii* infectivity of mammalian cell culture, suggesting they may be surface-exposed antigens. Further functional examination is required to validate the role of these proteins as rickettsial adhesins.

Sca PROTEINS AS VACCINE CANDIDATES

The immune responses generated from a prior rickettsial infection are seen to be protective and detectible for years following an infection (Bengtson and Topping, 1942; Fox et al., 1957). This has led to a hypothesis that the Sca proteins will provide attractive targets for recombinant vaccine production. There is currently no preventative therapy available for protection against rickettsial pathogens and antibiotic treatment with broad-spectrum antibiotics such as doxycycline, tetracycline, or chloramphenicol is effective only when administered within the first week of symptom manifestation, thus relying on prompt and proper diagnosis. *Rickettsiae* also pose a great biodefense threat and have historically been researched and employed as biological weapons (Harris, 1992; Kelly et al., 2002; Croddy et al., 2005).

Throughout the last 60 years, researchers have been exploring the potential use of whole bacterial immunization procedures through various treatments of the pathogens to prevent disease while maintaining the protective effects of exposure to the bacteria. Whole cell vaccines utilized in the early to mid-twentieth century against RMSF or typhus were comprised of formalin-fixed *R. rickettsii* or *R. prowazekii* isolated from ticks, embryonated chicken eggs, chick embryo fibroblasts, lice, or rabbit lungs. These vaccines failed to completely protect individuals from disease onset, but did reduce the severity

of the symptoms and facilitated an expedited response to antibiotic therapy (DuPont et al., 1973; Kenyon et al., 1975; Oster et al., 1977; Clements et al., 1983). Live attenuated strains of *R. prowazekii* (Madrid E and Δ*pld*) and *R. rickettsii* (Iowa) exist and have been demonstrated to be protective *in vivo* in animal models (Cox, 1941; Fox et al., 1957; Ellison et al., 2008; Driskell et al., 2009), and in the case of the *R. prowazekii* E strain, in human subjects as well (Fox et al., 1954, 1957).

Since $CD8^+$ T lymphocytes play a crucial role in the anti-rickettsial adaptive immune response and are critical effectors against intracellular infections, anti-*Rickettsia* vaccine development has focused heavily on understanding how to optimally stimulate cell-mediated immunity. Numerous studies have elucidated regions of *rompA* and *rompB* in DNA immunizations that exhibit the capacity to prime T cells for INF-γ production and cellular proliferation in response to stimulation with formalin-fixed rickettsial antigen preparations (Crocquet-Valdes et al., 2001; Diaz-Montero et al., 2001; Li et al., 2003). Although these regions are able to activate these requisite cell-mediated mechanisms *in vitro*, their protective effects *in vivo* have been limited. In these studies, complete protection of mice was achieved only with a combination of DNA/protein vaccinations (Crocquet-Valdes et al., 2001; Diaz-Montero et al., 2001). Interestingly, adoptive transfer of $CD8^+$ T cells from mice sub-lethally infected with *R. conorii* or *R. typhi* into naïve mice, fully protects mice against lethal homologous and heterologous rickettsial challenge (Valbuena et al., 2004), showing that effective priming of T lymphocytes can independently afford complete protection. Notably, the protection afforded by T lymphocytes was shown to be cross protective against divergent SFG and TG rickettsiae.

Humoral responses are believed to play a minor role in immune clearance of primary rickettsial infections, but instead contribute as a protective mechanism against re-infection. Several studies in mice and guinea pig animal models have examined the time-course of anti-SFG rickettsial antibody production in relation to disease resolution in sub-lethal rickettsial challenges, and determined that the antibody response matures after the primary infection has resolved, suggesting that the contribution of humoral immunity is in the prevention of secondary rickettsial infections (Lange and Walker, 1984; Feng et al., 2004a). Despite the minor role antibodies might play in resolving a primary rickettsial infection, anti-rickettsial antibodies have been demonstrated to perturb disease progression (Topping Norman, 1940; Anigstein et al., 1943; Feng et al., 2004a). Evidence from numerous studies indicate that antibodies against TG and SFG rickettsia species can facilitate bacterial clearance both *in vivo* and *in vitro* (Topping Norman, 1940; Anigstein et al., 1943; Gambrill and Wisseman, 1973; Beaman and Wisseman, 1976a,b; Lange and Walker, 1984; Anacker et al., 1985, 1987; Li et al., 1988; Keysary et al., 1989; Feng and Walker, 2003; Feng et al., 2004a,b). Humoral responses to live SFG rickettsial immunizations generate antibodies that specifically recognize high molecular weight rickettsial antigens (115–200 kDa), believed to be rOmpA and rOmpB based on SDS–PAGE migration, as well as protease-resistant species thought to be lipopolysaccharide (LPS) (Anacker et al., 1985; Li et al., 1988; Xu et al., 1997; Feng et al., 2004a). Characterization of monoclonal antibodies produced from these live rickettsial immunizations in various passive transfer studies have revealed that only a subset of antibodies recognizing epitopes belonging to the high molecular proteins afford protection, while those against LPS do not (Feng et al., 2004a). These findings led to the hypothesis that rOmpA and rOmpB could elicit protective immune responses in the host (Anacker et al., 1985, 1987; Li et al., 1988; Xu et al., 1997; Feng et al., 2004a); however, their roles as definitive protective antigens remain to be defined.

What may importantly be gleaned from these studies is that antibody recognition of single epitopes of single rickettsial antigens is potentially sufficient to neutralize rickettsial infection. In light of subsequent studies revealing the humoral immune-recognition of other high molecular weight Scas, including Sca1, Sca2, and Sca4 (Schuenke and Walker, 1994; Uchiyama et al., 1996; Uchiyama, 1997, and our unpublished data), it cannot be ruled out that the protective antibody responses attributed to rOmpA and rOmpB might also be associated with other less characterized rickettsial antigens. Notably, the humoral responses generated from a prior rickettsial infection have been seen to be long-lived and can be detected years following an infection (Bengtson and Topping, 1942; Fox et al., 1957). Though the robustness and longevity of anti-rickettsial humoral immunity must be examined in greater depth, these observations reasonably support the case that a vaccine comprised of one or more rickettsial antigens could be potentially efficacious. Further interrogation into the underlying features of rickettsial antigens necessary to elicit protective immunity is warranted.

PERSPECTIVE

Exploration of the contribution of various conserved Sca outer membrane proteins have led to the current model of rickettsial invasion of host cells (**Figure 3**). An overlapping but distinct set of proteins mediate adherence and/or invasion of these pathogens. These multiple interactions contribute to activation of an array of signaling pathways that ultimately recruit actin to the entry focus and result in uptake of the invasive *Rickettsia*. Undoubtedly, this model of invasion does not represent a complete picture of the host and bacterial factors involved in the entry process. However, the currently defined interactions serve as primary targets for therapeutics. Through detailed biochemical and genetic analyses these host–pathogen interactions may be inhibited, thus resulting in a loss of bacterial invasion and death of the pathogen.

A fact to be noted is the extraordinary amount of effort and creativity that was put into the above described research. The development of methods for selective deletion of rickettsial genes (Driskell et al., 2009) allows for direct examination of the contribution of adherence and invasion genes on pathogenesis of these bacteria. As it appears that many different rickettsial proteins contribute to invasion of host cells (Li and Walker, 1998; Renesto et al., 2006; Uchiyama et al., 2006; Cardwell and Martinez, 2009; Chan et al., 2009; Riley et al., 2010), it seems plausible that any single gene encoding these functions may be successfully deleted. These analyses will provide great insight into rickettsial diseases including the relative contribution of each protein to pathogenesis, their roles in disease progression or resolution, potential organ tropism, stimulation of host cellular signaling, and so forth. Determination of rickettsial factors and host proteins involved in *Rickettsia* interactions with target cells will provide valuable information both into the pathogenic process and also serve as prime targets for interventional therapy.

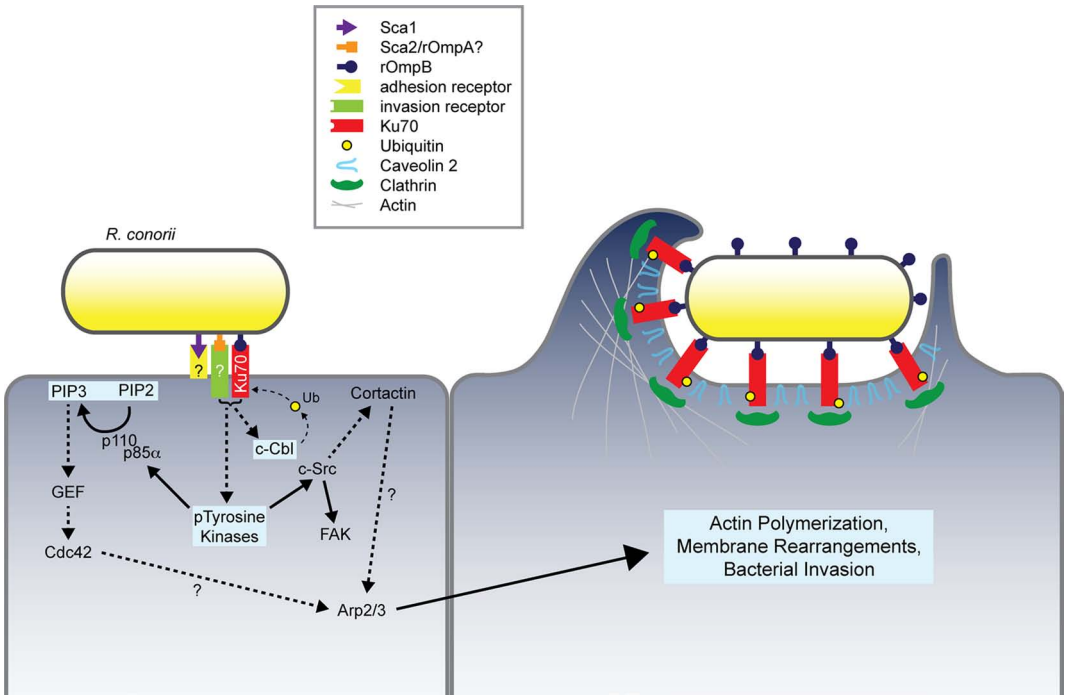

FIGURE 3 | Model of *Rickettsia conorii* invasion of non-phagocytic mammalian cells. *R. conorii* interaction with host receptors, including Ku70, activates numerous signaling pathways whose coordinated activities lead to modulation of the actin cytoskeleton and predicted localized recruitment of endocytic players including clathrin and caveolin 2. *R. conorii* internalization depends on the stimulation of multiple pathways involving PTKs, Cdc42, PI 3-kinase, Src, FAK, c-Cbl, and cortactin, which ultimately result in Arp2/3-dependent actin polymerization at the bacterial entry foci. Pathways and proteins involved in rOmpB–Ku70-mediated bacterial invasion have been highlighted in light blue boxes. Dashed arrows and question marks indicate putative protein interplay during the invasion process.

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Systemic effector and regulatory immune responses to chlamydial antigens in trachomatous trichiasis

Alevtina Gall[1], Amir Horowitz[2], Hassan Joof[1], Angels Natividad[3], Kevin Tetteh[4], Eleanor Riley[2], Robin L. Bailey[3], David C. W. Mabey[3] and Martin J. Holland[1,3]*

[1] *Viral Diseases Programme, Medical Research Council Laboratories, Banjul, The Gambia*
[2] *Department of Immunology and Infection, London School of Hygiene and Tropical Medicine, London, UK*
[3] *Department of Clinical Research, London School of Hygiene and Tropical Medicine, London, UK*
[4] *Department of Pathogen Molecular Biology, London School of Hygiene and Tropical Medicine, London, UK*

Edited by:
Rey Carabeo, Imperial College London, UK

Reviewed by:
Kathleen Kelly, University of California at Los Angeles, USA
Toni Darville, University of Pittsburgh Medical Center, USA

***Correspondence:**
Martin J. Holland, Department of Clinical Research, London School of Hygiene and Tropical Medicine, London WC1E 7HT, UK.
e-mail: martin.holland@lshtm.ac.uk

Trachomatous trichiasis (TT) caused by repeated or chronic ocular infection with *Chlamydia trachomatis* is the result of a pro-fibrotic ocular immune response. At the conjunctiva, the increased expression of both inflammatory (*IL1B, TNF*) and regulatory cytokines (*IL10*) have been associated with adverse clinical outcomes. We measured *in vitro* immune responses of peripheral blood to a number of chlamydial antigens. Peripheral blood effector cells (CD4, CD69, IFNγ, IL-10) and regulatory cells (CD4, CD25, FOXP3, CTLA4/GITR) were readily stimulated by *C. trachomatis* antigens but neither the magnitude (frequency or stimulation index) or the breadth and amount of cytokines produced *in vitro* [IL-5, IL-10, IL-12 (p70), IL-13, IFNγ, and TNFα] were significantly different between TT cases and their non-diseased controls. Interestingly we observed that CD4+ T cells account for <50% of the IFNγ positive cells induced following stimulation. Further investigation in individuals selected from communities where exposure to ocular infection with *C. trachomatis* is endemic indicated that CD3–CD56+ (classical natural killer cells) were a major early source of IFNγ production in response to *C. trachomatis* elementary body stimulation and that the magnitude of this response increased with age. Future efforts to unravel the contribution of the adaptive immune response to conjunctival fibrosis should focus on the early events following infection and the interaction with innate immune mediated mechanisms of inflammation in the conjunctiva.

Keywords: ***Chlamydia trachomatis*, trachoma, immune response, Tregs, NK cells, interferon-gamma**

INTRODUCTION

Chlamydia trachomatis is the leading infectious cause of blindness worldwide. Persistent infection or recurrent infection with *C. trachomatis*, can stimulate a fibrotic immune response that leads to deposition of scar tissue on the upper tarsal conjunctiva and the development of a dry eye syndrome. The progressive development of scar tissue can lead to entropion and trachomatous trichiasis (TT), which if left untreated may result in damage to the cornea causing opacity, low vision, and blindness (Mabey et al., 2003). There are an estimated eight million individuals currently suffering from TT with about 1.3 million irreversibly blind as a result (Mariotti et al., 2009).

Chlamydia trachomatis is also the most common bacterial sexually transmitted infection. It is frequently asymptomatic, especially in women and can lead to sub-fertility, chronic pelvic inflammatory disease, ectopic pregnancy, and sterility (WHO, 2007). Although both ocular and genital chlamydial infections are treatable with antibiotics, persistent, and recurring infection is common. A licensed vaccine against *C. trachomatis* for use in the human population is not yet available despite continued efforts since the 1960s. Recent developments (reviewed by Rockey et al., 2009) have yielded encouraging results in some animal models; however, the development of an effective vaccine requires further understanding of the basic features of the human immune response to *C. trachomatis* infection. Indeed work in mice has suggested that chlamydial vaccine induced immunity without pathology can be achieved and this is distinct from immunity induced by infection that results in pathology (Igietseme et al., 2009). However in naturally exposed populations only a minority develops the pathological sequelae of infection, suggesting that the immunological features of naturally immune individuals may be equally distinct.

In animal models of infection it is well established that CD4+ T helper type 1 (Th1) cells are essential for the clearance of primary chlamydial infection (Igietseme et al., 2009). In particular, IFNγ plays an important role in the clearance of chlamydial infection. In mice and in humans its production is associated with protection from re-infection (Wang et al., 1999; Cohen et al., 2005). However, uncontrolled inflammatory responses, in part driven by IFNγ production, result in pathology. Regulatory T cells (Tregs), induction of regulatory cytokines such as IL-10 or the activation of type 2 (Th2) responses counteract the inflammatory environment and contribute to restoring homeostasis (Yang et al., 1999; Wang et al., 2005). Conversely unchecked type 2 responses are frequently associated with chronic inflammation and infection (Wynn, 2004) and hampering Th2 responses have been implicated in chlamydial infections and induced disease (Holland et al., 1993, 1996; Gondek et al., 2009). In addition some immune mediated fibrotic diseases, such as schistosomula induced hepatic fibrosis,

are also dominated by Th2-cytokine responses (Chiaramonte et al., 2003). The pathogenic changes observed at the site of infection in chlamydial genital and ocular disease are also due to development of fibrotic tissue yet convincing evidence of a significant role for polar CD4+ Th2 responses or alternative macrophage activation are lacking (Holland et al., 2010; Natividad et al., 2010). Many groups have now demonstrated the important role of immunoregulatory subsets of CD4+ T cells in preventing immune damage during viral, bacterial, and parasitic infections, and specifically in persistent infections at mucosal surfaces (Maloy and Powrie, 2001; Belkaid and Tarbell, 2009) but these have not been extensively studied in human chlamydial disease.

One subset of CD4+ T cells that has key functions in immune homeostasis are Tregs (CD4+CD25+FOXP3+). The regulatory function of these natural and adaptive Tregs may be mediated by production of TGF-β and IL-10 or by cognate interaction (Hori et al., 2003; Fontenot et al., 2005). Antigen-specific IL-10 secreting type-1 T regulatory cells (T_R1) which do not express *FOXP3* have also been described *in vivo* following chronic antigenic stimulation (Vieira et al., 2004), and develop in parallel with Th1 cells in several chronic infectious diseases (McGuirk et al., 2002). Murine studies have established that *FOXP3* + regulatory cells can secrete both IFNγ and IL-10 (Stock et al., 2004) and in humans regulatory function can also be exerted via perforin dependent lysis (Grossman et al., 2004). Thus, although most work has focused on cells with a classic Treg phenotype (CD4+CD25+FOXP3+), cells from CD4+CD25–, CD8+, and NKT subsets can all exert regulatory function (Jiang and Chess, 2004). There is also evidence that CD4+ Th1 cells can exhibit self-regulatory properties and cytokine profiles, obviating the need to explain disease in terms of a separate regulatory class of T cells (Anderson et al., 2007; Jankovic et al., 2007).

We have previously demonstrated a relative *Chlamydia*-specific hypo-responsiveness associated with chronic ocular infection (Holland et al., 1993; Bailey et al., 1995), and described imbalances in antigen-specific secretion of both IL-10 and IFNγ in cultured peripheral blood mononuclear cell (PBMC) from scarred subjects (Holland et al., 1996). We have also found that IFNγ, IL-10, and perforin expression in the conjunctiva is associated with the clinical signs of active trachoma (Burton et al., 2004); that susceptibility to scarring sequelae is affected by haplotypic variation at the IL-10 locus (Natividad et al., 2005, 2008), that contributes to variation in IL-10 expression; and that the expression of *FOXP3* is up-regulated in individuals who have recently resolved ocular *C. trachomatis* infection (Faal et al., 2006).

In animal models of viral ocular infection, several groups have identified an important role for Tregs in modulating the severity of disease (Suvas et al., 2004; Nesburn et al., 2007). Aside from FOXP3, other potential makers of regulatory T cells have been identified in the search to phenotypically define Tregs. Most commonly the relative expression of the IL-7 receptor (CD127) is used as a fourth surface marker (Finney et al., 2010), the low expression of which is regarded as further evidence of Tregs. Two other common markers with demonstrated independent down regulatory properties are Cytotoxic T Lymphocyte Antigen 4 (CTLA4) and Glucocorticoid-induced tumor necrosis factor-receptor related protein (GITR) both of which have been linked to the suppressive mechanism of Tregs (Zheng et al., 2004; Nocentini and Riccardi, 2005). Moreover, in rabbits with ocular HSV infection, cells in the conjunctiva, which expressed high levels of FOXP3, GITR, and CTLA4, were effectively able to suppress the effector function of HSV-specific CD4+ and CD8+ T cells (Nesburn et al., 2007).

In this study we compared the *in vitro* immune responses of individuals with TT and controls with normal eyes from trachoma endemic communities, since we expected that immune responses from these individuals would be the most polar. We investigated the immune responsiveness of several chlamydial antigens that had not previously been measured in individuals from trachoma endemic communities. Cytokine levels in supernatants from proliferation assays were quantified for IL-5, IL-10, IL-12p70, IL-13, IFNγ, and TNFα. Immune responses were also assessed using flow cytometry, where cells were stained for IFNγ and IL-10, as well as Treg cell markers FOXP3, CTLA4, and GITR. To investigate which cells were the major sources of IFNγ, we further stained for markers that included CD8 and CD3–CD56+ cells in PBMC isolated from young and adult individuals from the same trachoma endemic environment.

MATERIALS AND METHODS

STUDY PARTICIPANTS

Informed consent was obtained from all study participants. Participants were recruited from rural and semi-urban areas within the Western Region and the Lower river regions (LRR) of The Gambia. Trachoma was graded using the WHO simplified grading system by a single experienced field supervisor. Forty-two subjects with TT (more than one eyelash touching the globe of the eye) were identified. For each TT case an age, sex, and location matched control subject with normal eyes that was not a member of the same family was also recruited. Participants were age matched within 10 years. In a standardized manner, an ocular swab from the everted tarsal conjunctiva of each participant was collected into RNA*later*® (Ambion Ltd) for the later isolation of proteins and nucleic acids. For the *in vitro* study of natural killer (NK) cell responsiveness, five healthy participants from three different age groups (2–5; 15–25; >35 years) from trachoma endemic communities in the West Kiang District of LRR were recruited. Historical exposure to ocular infection in these districts was expected to be high since structured surveys as part of a national trachoma control program identified this district as requiring mass drug treatment. As a result, these communities were treated with three annual rounds of oral azithromycin, completed in 2009 as per WHO guidelines for community control strategies. The joint Scientific and Ethics Committee of the Gambian government and the Medical Research Council Laboratories approved each study (applications L2006.10 and SCC1201). In addition the ethics committee of the LSHTM also approved the studies. All study participants outside of community trachoma control programs were offered treatment according to national eye care program guidelines.

PROLIFERATION ASSAYS

Venous blood (20 ml) was collected into tubes containing 300 units of sterile, preservative-free sodium heparin. After the removal of 2.5 ml of blood for whole blood cultures the PBMCs were isolated from the remaining blood by centrifugation over Lymphoprep (Axis-Shield Ltd, Kimbolton, UK) as described previously (Holland et al., 1996).

Plasma was harvested by collection from above the PBMC layer and stored at −30°C for later use in enzyme-linked immunosorbent assays (ELISA) to estimate the anti-chlamydial antibody titer. PBMCs were co-incubated for 7 days with 5 μg/ml of each antigen (Pokeweed mitogen, PWM; Purified protein derivative, PPD) of *Mycobacterium tuberculosis* strain Rv37 and the following *C. trachomatis* antigens; Serovar A (strain SA1) elementary bodies (EBs), major outer membrane protein (MOMP), Enolase, polymorphic outer membrane protein (Pmp) D, PmpG, outer membrane protein (Omp) 2. On day six, 100 μl of culture supernatant was removed from each well without disturbing the cell pellet and 1 μCi/well ^{3}H-thymidine added for the last 18 h of culture. The culture supernatant was frozen at −30°C until tested by multiplex bead array for cytokines. ^{3}H-thymidine incorporation was estimated using liquid scintillation counting (Micro-beta, Wallac, Turku, Finland). Results were expressed as $\log_{10}$ stimulation indices (SI), which were calculated as described by Bennett and Riley (1992) using the geometric mean of the triplicate counts per minute (CPM) for the test antigen/CPM (geometric mean) of triplicate wells that only received cells and RPMI.

CYTOKINE DETECTION

The triplicate wells of day six supernatants from microtiter plates were pooled and 25 μl used in a six-plex (IL-5, IL-10, IL-12p70, IL-13, IFNγ, and TNFα) cytokine assay (Bio-Rad, United Kingdom) according to the manufacturer's instructions. The plates were then read using a Bio-plex instrument (Bio-Rad, UK). Unknown samples were quantified by reading standard curves prepared for each of the cytokines under study. Lower and upper limits of detection were dependent on assay performance. Any samples with cytokine levels that were outside of the working range of each assay were reported as under or over the detection limit and for the purposes of analysis were given a value equal to the minimum or maximum detectable level.

ANTIBODY STAINING AND FACS ANALYSIS

Five hundred microliters of whole blood was co-stimulated with 10 μg/ml of FastImmune™ [CD28/CD49d co-stimulatory reagent (BD Biosciences)] and incubated with 5 μg/ml of whole Ct EB or *Staphylococcus aureus* Enterotoxin B (SEB) for 48 h. Cultures stimulated with FastImmune™ alone served as controls. BD GolgiPlug™ (Brefeldin A; BD Biosciences) was added for the last 18 h of culture at 1 μg/ml. Cells were harvested by centrifugation followed by lysis of red blood cells and further washing in serum free RPMI by centrifugation. When required, PBMC were permeabilized for intracellular staining. The following antibodies were used: CD4-PerCP (SK3), CD25-FITC (M-A251), IFNγ-FITC (4S.B3), IL-10-PE (JES3-19F1) and CTLA4-PE (BNI3) CD69-APC (FN50; BD Pharmingen), FOXP3-APC (PCH101; eBiosciences), and GITR-PE (110416; R&D Systems). Samples were then examined using a FACS Caliber flow cytometer (Becton, Dickinson and Company, NJ, USA) and the data collected using CellQuest-Pro. Files were further analyzed using FlowJo software (Treestar, OR, USA).

CELL SURFACE AND INTRACELLULAR STAINING FOR NK CELLS

Surface and intracellular staining was performed as described previously (Horowitz et al., 2010a,b). Briefly 4×10^5 PBMC were incubated for 24 h with 10 μg/ml EBs. As a positive control rIL-12/IL-18 were added to separate cultures. Abs used were as follows: CD69 PE (CH/4; BD Pharmingen), CD56 PE (N901), CD56 APC (N901; both Beckman Coulter, Fullerton, CA, USA), CD56 PE-Cy7 (B159), CD3-PerCP (SK7), CD4 APC-Cy7 (RPA-T4), CD4 PE (RPA-T4), CD8 PE (SK1), CD3 PE-Texas Red (S4.1) CD107a (LAMP-1) biotin (H4A3; Caltag/Invitrogen), CD8 Pacific Blue (LT8; eBiosciences, San Diego, CA, USA), and IFNγ FITC (D9D10; Ab Serotec). For CD107a staining antibody and GolgiPlug™ were added to the cultures for the last 4 h of stimulation when they were washed and permeabilized. The remaining cocktail of antibodies was then added staining both surface and intracellular antigens. Samples were then examined using a Dakocytomation CyAn flow cytometer (Beckman Coulter, Fullerton, CA, USA) and the data collected using Summit FCS 4.3 software. Files were further analyzed using FlowJo (Treestar, OR, USA).

ISOLATION OF DNA FROM OCULAR SWABS

DNA was isolated from ocular swabs using Qiagen RNeasy micro kits and DNA mini kits as described elsewhere (Holland et al., 2010). The presence of *C. trachomatis* was then tested using CT/NG by Amplicor (Roche) as described previously (Holland et al., 2010). Sample integrity and quality for each ocular swab was confirmed by performing PCR on genomic DNA using primers for human-specific hypervariable 1 (HV1) D-loop region mitochondrial DNA as described previously (Harding-Esch et al., 2009a).

SEROLOGY

Plasma antibody titrations were performed using purified serovar A EBs immobilized on ELISA microtiter plates as described previously (Wang et al., 2009, 2010).

STATISTICAL ANALYSIS

Statistical analysis was performed using the statistical analysis tool in GraphPad Prism 5 software (GraphPad Software, Inc, La Jolla, CA, USA). A paired Student's *t*-test was used for combined data of cases and controls when compared to background controls. When comparing the response of matched cases and controls an adaptation of the Mann–Whitney distribution-free paired test was used (Wilcoxon signed rank). For the simultaneous comparison of three age groups we used a non-parametric ANOVA (GraphPad Prism) which is a omnibus Kruskal–Wallis test progressing to *post hoc* testing of simultaneous pairwise comparisons using Dunn's Test if the overall P-value < 0.05. Both Kruskal–Wallis and Dunn's Test are distribution-free tests of significance.

RESULTS

STUDY PARTICIPANTS

The basic study participant details from each section of the study are described in **Table 1**. Forty-two case–control pairs were used in the lymphocyte proliferation assays. Up to 17 of these case–control pairs were studied using FACS analysis and multiplex bead arrays for cytokines. Approximately 2/3 of the case–control pairs were from females (27/42) and the median age was 58 years. All ocular swabs for these samples were positive for human mitochondrial DNA and negative by CT/NG Amplicor for current ocular infection

Table 1 | Study participant demographics and Ct antibody titers.

PBMC PROLIFERATION SAMPLES			
	Cases (*n* = 42)		**Controls (*n* = 42)**
Age (range) in years	60 (30–85)		57 (30–80)
Sex			
Male	15		15
Female	27		27
Anti-Ct (serovar A) antibody titer	1×10^4 ($<1 \times 10^3$–2×10^4)		7.5×10^3 ($<1 \times 10^3$–2×10^4)
CT/NG Amplicor	0		0
FLOW CYTOMETRY AND BIO-PLEX			
	Cases (*n* = 17)		**Controls (*n* = 17)**
Age (range) in years	58 (35–70)		54 (31–75)
Sex			
Male	6		6
Female	11		11
Anti-Ct antibody titer (1/×; range)	1×10^4 ($<1 \times 10^3$–2×10^4)		1×10^4 ($<1 \times 10^3$–2×10^4)
CT/NG Amplicor (*n* = no. positive)	0		0
NK PBMC FLOW CYTOMETRY			
	<5 years (*n* = 5)	**<25 years (*n* = 5)**	**>35 years (*n* = 5)**
Age (range)	3 (2–5)	17 (16–25)	67 (37–72)
Sex			
Male	3	3	2
Female	2	2	3
Anti-Ct (serovar A) antibody titer	1×10^3	1×10^3 ($<1 \times 10^3$–2×10^4)	1×10^4 ($<1 \times 10^3$–2×10^4)
No. with clinical signs	0	0	0
No. seropositive	2	5	5
CT/NG Amplicor (*n* = no. positive)	n.t.	n.t.	n.t.

with *C. trachomatis*. Plasma anti-*C. trachomatis* antibody levels were not significantly different between the clinical groups and all sera were positive compared to plasma from immunologically naïve individuals.

LYMPHOCYTE PROLIFERATIVE RESPONSES TO *C. TRACHOMATIS* ANTIGENS

To determine whether the proliferative response of TT cases differed from that of the controls we studied the responses to chlamydial EBs and five recombinant antigens (**Table 2**). PWM was used as a positive control for the stimulation of T and B cells. We considered a SI > 2 as a significant proliferative response. Enolase, PmpD, PmpG, and Omp2, induced a pathogen-specific proliferative response that was above background and comparable to proliferation induced by stimulation with EBs. In general and as a trend, responses were higher in cases compared to controls; however, no statistically significant differences were found between TT cases and controls (**Figure 1**).

STIMULATION OF CYTOKINE RESPONSES BY *C. TRACHOMATIS* ANTIGENS

Day six supernatants from proliferation assays were collected and assessed for the presence of IL-5, IL-10, IL-12p70, IL-13, IFNγ, and TNFα cytokines. All antigens tested were able to elicit an IL-10, IL-12p70, IFNγ, and TNFα cytokine response that was significantly above background. Few antigens, however, were able to stimulate IL-5 or IL-13 production. The levels of antigen-specific cytokine production are summarized in **Figure 2**. EB stimulated responses were significantly above background for IL-10, IFNγ, and TNFα in both TT cases and controls. However, these cytokine profiles did not correlate with disease status (**Figure 2**). Taken together the cytokine and proliferation data show that peripheral blood proliferative responses and cytokine production profiles are not significantly different between participants with and without TT.

CD4+CD25+FOXP3+ CELLS ARE INCREASED FOLLOWING STIMULATION WITH *C. TRACHOMATIS* ANTIGENS

We investigated whether immune responses of TT patients differ in regulatory phenotypic markers when stimulated by chlamydial antigens compared to their respective controls. Whole blood from 17 case–control pairs ($n = 34$) was stimulated with *C. trachomatis* EBs for 48 h and stained with antibodies which in combination identify Tregs (CD4+CD25+FOXP3+). FOXP3+ cells were largely contained in the CD4+CD25+ population, where the CD25+ population was continuous and no distinction was made between $CD25^{hi}$+ and $CD25^{lo}$+ cells. EBs were particularly effective at inducing Tregs, relative to those cells that received co-stimulation alone (**Figure 3A**). **Figure 3B** shows an example of the flow cytometry multilevel gating strategy used on each sample. CTLA4 and GITR were also used as additional surface markers of Treg activity. Stimulation with chlamydial EBs was effective in inducing the up-regulation of CTLA4 and GITR (**Figure 3B**).

Table 2 | Summary of recombinant antigens used.

Serovar D locus-tag (NC000117)	Name	Antigen details (kDa)	Serovar origin	DNA insert fragment size (bp)	Product size by SDS–PAGE (kDa)	Production of original expression clone	Fusion protein type	Recombinant expression and purification	Publications (cellular responses only)
CT812	PmpD	Polymorphic outer membrane protein (polymorphic family of 9 genes A–I; 160)	L2	2.024 kb (690–2714 gene fragment)	74	Prof. Hill Gaston and Dr. Jane Goodall, University of Cambridge	His-tag	pPET/ProBond®	Goodall et al. (2001b)
CT681	MOMP	Major outer membrane protein (40)	L1	1.6 kb (285–1182 gene fragment)	33	Prof. Ian Clarke, University of Southampton	B-gal	Induced insoluble inclusions purified by centrifugation resuspended in octylglucoside/ DTT	Holland et al. (1993)
CT871	PmpG	Polymorphic outer membrane protein (107)	A	1.509 kb (127–1726 gene fragment)	61	Dr. Mark Felton, LSHTM	His-tag	pPET/ProBond®	Felton (2002)
CT587	Enolase	Phosphopyruvate hydratase (enolase family; 45)	L2	1.274 kb (full length)	45	Prof. Hill Gaston and Dr. Jane Goodall, University of Cambridge	His-tag	pPET/ProBond®	Goodall et al. (2001b)
CT443	Omp2	Cysteine rich outer membrane protein (60)	L2	1.65 kb (700–2350 gene fragment)	60	Prof. Hill Gaston and Dr. Jane Goodall, University of Cambridge	His-tag	pPET/ProBond®	Goodall et al. (2001a)

However, when these phenotypic markers of regulatory activity were compared between TT cases and their controls, no statistical difference was found (**Figure 3C**). As a general trend CD4+CD25+FOXP3+ cells and CD4+GITR+ cells were higher in TT cases to all chlamydial antigens tested (additional data not shown). Similarly, no difference was detected in the frequency of CD4+FOXP3+ or CD4+CD25+ cells between cases and controls. Since no differences in regulatory cells were apparent we also used the CD4+CD25+FOXP3− population as an indicator of effector cell responses and found that the population frequencies of these cells were equivalent between cases and controls stimulated with EBs (**Figure 3C**).

ACTIVATED LYMPHOCYTES PRODUCE IFNγ AND IL-10 IN RESPONSE TO *C. TRACHOMATIS* ELEMENTARY BODIES

Whole blood was stimulated as previously described and stained for CD69, a marker of early activation, in addition to CD4, intracellular IFNγ and IL-10. Stimulation with EBs showed a marked increase in CD69 expression and a higher frequency of IFNγ and IL-10 producing cells. **Figure 4A** shows the FACS analysis gating procedure used to examine IFNγ and IL-10 production from CD4+ T cells and total PBMC. Despite the induction of CD69 on CD4+ cells and the production of IFNγ and IL-10, effector T cell responses were not significantly different between cases and controls (**Figure 4C**).

CD4 + CELLS ARE NOT THE MAJOR IFNγ PRODUCING CELLS IN RESPONSE TO STIMULATION WITH *C. TRACHOMATIS* ELEMENTARY BODIES

To assess if CD4+ T cells encapsulated the observed increases in the percentage of CD69, IFNγ, and IL-10 in the total stimulated population of peripheral blood lymphocytes we examined the percentage of each of these in the CD4+ compartment and the total PBMC compartment. We found that on average less than half of the cells that were producing IFNγ, IL-10, or expressed

CD69 were also CD4+ (**Figure 4B**). To establish which cells were responsible for IFNγ production we tested PBMC from individuals exposed to ocular infection but without current signs of trachoma. Five individuals were selected from three different age ranges. This strategy was chosen because we had demonstrated that endemic controls had significant responses to EBs above those observed with co-stimulation alone, but that these were not significantly different between case and control participants. **Figure 5A** shows representative FACS plots and flow diagrams of the gating and staining protocols used. There was some natural variation with age in the total percentage of NK cells (CD3−CD56+ cells) and of CD8+ T cells. The percentage of CD4+ T cells did not appear to vary with age (**Figure 5B**). We found that in the first 24 h of *in vitro* stimulation, EBs induced IFNγ in CD4+, CD8+, and NK cells. The percentage of IFNγ producing cells in response to stimulation with EB significantly increased with age and adults over 35 years of age demonstrated the highest levels. The percentage of CD4+ IFNγ+, although increased, did not appear to vary with age and the overall levels were approximately 4× lower. Interestingly, CD8+ IFNγ+ T cells were highest in the first two age groups though the overall levels were lower than those observed for NK (CD3−CD56+) cells (**Figure 5C**). Further confirmation that EB stimulation resulted in stimulation of NK cells that increased with age was seen by increasing levels of CD107a+ NK cells, whereas CD8+CD107a+ T cells decreased with age or were almost absent. As expected the CD4+ T cells expressed negligible levels of CD107a (**Figure 5D**). **Figures 5C, D** further show that stimulation with EB leads to increases in the percentages of both IFNγ and CD107a expressing NK cells (up to 40% of NK cells express CD107a when stimulated by EB in adults over 35 years of age) compared to control cultures with culture medium alone and that in each case the difference and magnitude increases with age.

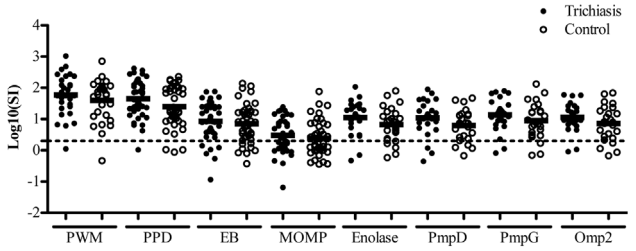

FIGURE 1 | Trachomatous trichiasis cases do not differ in proliferative responses to *Chlamydia trachomatis* antigens compared to controls. PBMCs $\log_{10}$ stimulation index (test antigen c.p.m/non-stimulated c.p.m) were calculated after 6 days of stimulation with PWM or chlamydial antigens EBs, MOMP, Enolase, PmpD, PmpG, Omp2. Trichiasis cases (•) and disease free endemic controls (○). Paired students *t*-test was performed on each group (up to 42 pairs). The dashed line represents stimulation index = 2. All responses above this dashed line were considered above background. Each antigen induced a statistically significant response above background. No statistical significance was detected between proliferative responses of the clinical groups to any chlamydial antigen. Analysis based on the proportion of responder (SI > 2) versus non-responder (SI < 2) also found that there were no differences evident between the clinical groups.

DISCUSSION

Both TT cases and controls had lymphoproliferative responses that were above background when stimulated with EBs, Enolase, PmpD, PmpG, and Omp2 antigens. Responses to some of these antigens have been investigated as putative vaccine candidates (Coler et al., 2009; Goodall et al., 2001a,b) and studied in human infection. More recently, a number of chlamydial antigens such as LcrE (CPn0324/CT089) and chlamydial protease-like activating factor (CPAF/CT858) have been proposed as potential vaccine candidates and their responsiveness has been tested in animals and humans (Olsen et al., 2006, 2007, 2010; Thorpe et al., 2007; Barker et al., 2008; Follmann et al., 2008). Although most of the TT study participants were able to elicit proliferative responses to the chlamydial antigens, the degree of proliferation was not significantly different between individuals with severe scarring disease and controls without disease. As a general trend responses to each antigen were higher in cases with TT, in contrast to our previous findings (Holland et al., 1993, 1996). However, in previous studies higher proportions of individuals with trachomatous scarring were still infected with *C. trachomatis* and were at earlier stages of disease, where *C. trachomatis* driven fibrotic responses could still be progressing. We also previously found that serum antibody responses in those with trachomatous scarring were higher compared to controls whereas in this cohort *C. trachomatis* serovar A antibody responses were equal between the groups. This suggested that both groups of individuals had experienced similar levels of exposure to ocular infection and that there had been little recent infection. This changing environment could explain why we did not detect any significant differences compared with our own previous findings (Harding-Esch et al., 2009b).

Although MOMP constitutes up to 60% of an EB it nevertheless contains a significant proportion of the total antigenic repertoire (Wang et al., 2010). It is the most abundant surface exposed protein of EBs, is highly conserved across *C. trachomatis* serovars containing a large number of T cell specific epitopes (Kim and DeMars, 2001; Nunes et al., 2010) and anti-MOMP antibodies have been shown to neutralize *C. trachomatis in vitro* (Cotter et al., 1995). Because of these properties it has been widely studied as a putative vaccine candidate (Zhang et al., 1997). More recently native MOMP (nMOMP) has been used to immunize monkeys and successfully induced high titers of neutralizing antibodies that reduced the infectious ocular burden within the first 2 weeks of infection. Despite the promising results nMOMP immunization had no lasting effects on the course of clinical disease or burden and duration of infection (Kari et al., 2009). In our experiments MOMP induced only weak responses in the majority of individuals. Antibodies specific to PmpD have also been shown to neutralize *C. trachomatis in vitro* regardless of serotype (Crane et al., 2006), and Omp2 has been shown to be recognized by *Chlamydia*-specific human CD8+ T cells (Gervassi et al., 2004). PmpG which is part of the outer membrane complex and may be involved in the invasion of the host cell by *C. trachomatis* was also tested. Each of these antigens induced responses but these were not significantly different between cases and controls. Thus far, with the exception of MOMP and hsp60, no other antigens have been evaluated by measuring cell mediated immune responses in individuals with trachoma. Skwor et al. (2010) have tested human tear fluid for

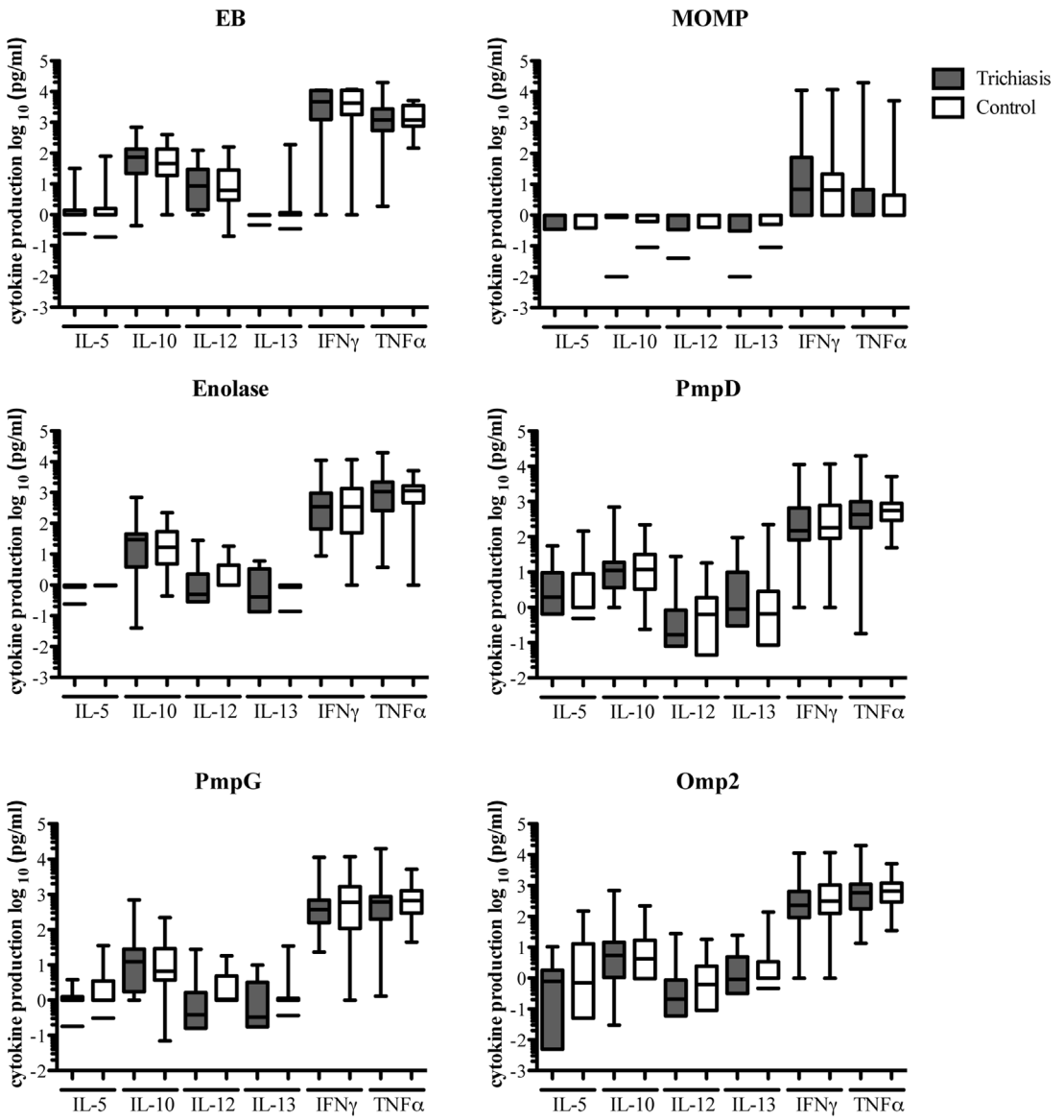

FIGURE 2 | Chlamydial antigens induce cytokine responses that are significantly above responses of cultures in culture medium alone, however, the levels of cytokine production are not statistically different between diseased and non-diseased individuals. Each plot represents IL-5, IL-10, IL-12p70, IL-13, IFNγ and TNFα production in response to stimulation with *Ct* EBs, MOMP, Enolase, PmpD, PmpG, and Omp2. Responses from TT cases and controls are depicted as shaded and non-shaded box and whisker plots respectively.

antibody reactivity to a number of antigens (cHSP60, CPAF, and CT795) and found that IgG responses to CPAF were associated with TT cases and responses to all three antigens were elevated during inflammation, but it remains unclear how this might relate to cellular responses and requires further investigation.

Interleukin-10 and IFNγ have been extensively studied in mice and humans with respect to chlamydial infection and IL-10 has been found to be associated with the persistence of infection, while IFNγ has been linked to its resolution (reviewed in Yang, 2003). One study using IFNγ knockout mice infected with *C. muridarum* found that production of IL-5 was elevated and correlated with more severe disease (Wang et al., 1999). Another study found that when murine dendritic cells were infected with *C. trachomatis,* the subsequent production of IL-12 and TNFα correlated with containment of the bacteria (Knight et al., 1995). Furthermore, studies have shown that mice infected with *C. muridarum* that received CD11c+ CD8α+ cells had increased amounts of proinflammatory cytokines such as IFNγ, TNFα, and IL-12 and decreased production of the Th2 cytokines IL-4, IL-5, and IL-13, which correlated with protection against infection. Our results show that in peripheral blood of individuals exposed to *C. trachomatis,* most chlamydial antigens tested were able to produce above background levels of IL-10, IL-12, IFNγ, and TNFα cytokines

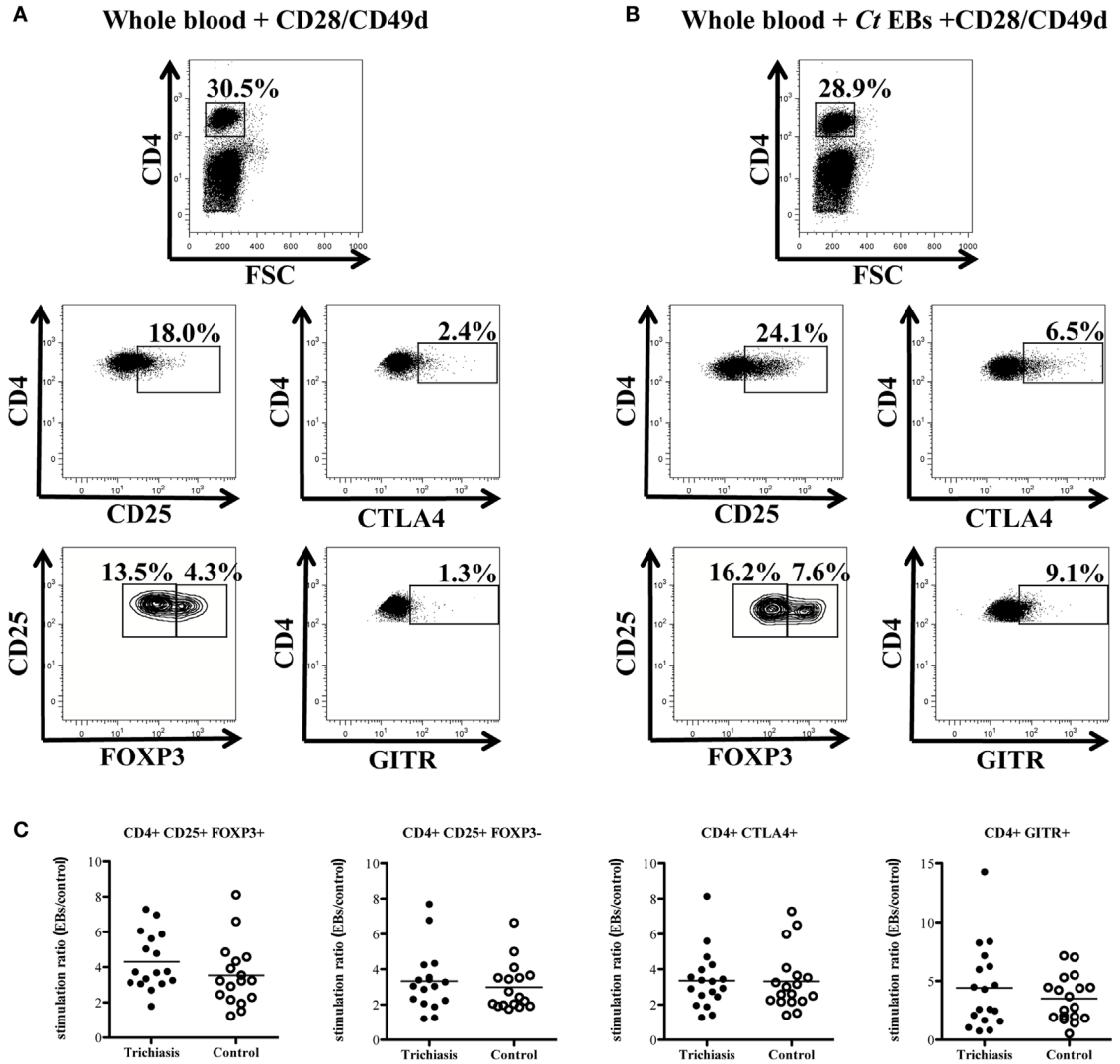

FIGURE 3 | Representative FACS plots of whole blood cultured for 48 h with co-stimulatory molecules CD28/CD49d (A) or CD28/CD49d and chlamydial elementary bodies **(B)** are shown to demonstrate the gating strategy used to enumerate the percentage of Tregs. FOXP3+ cells were gated within the population of CD4+CD25+ cells. The CD25+ population was taken as continuous ($CD25^{hi}$ and $CD25^{lo}$ expressing cells were not differentiated). CD4+ effector cells were identified as the CD4+CD25+FOXP3– population. **(C)** Responses of trichiasis cases (n = 17) and endemic controls (n = 17) are represented as a stimulation ratio (i.e., percentage of CD4+CTLA4+ cells induced by EB stimulation/percentage of CD4+CTLA4+ induced by co-stimulation alone). Tregs and CD4+GITR+ cells were higher in trichiasis cases but there were no statistically significant differences between cases and controls in either regulatory or effector T cell populations.

after 6 days of culture. Only PmpD and Enolase were able to stimulate IL-5 and IL-13 cytokine responses respectively. However, there were no clear indications that any particular antigen or cytokine profile was significantly different between cases and controls. This suggests that a larger population study is required with follow-up and repeat testing, or that the methods employed *in vitro* are not sufficiently sensitive to detect differences in these particular cytokines.

Whilst the roles of IL-10 and IFNγ have been intensively studied in chlamydial disease, there has been little investigation of regulatory T cells in mediating the response to *C. trachomatis*. Our results show that Tregs (CD4+CD25+FOXP3+) and the co-stimulatory surface markers with inhibitory or regulatory functions (CTLA4 and GITR) are up-regulated in response to stimulation with chlamydial EBs. We also found higher levels of CD4+CD25+FOXP3+ and CD4+GITR+ T cells in TT cases compared to controls (although these differences were not statistically significant). Other authors have suggested that the CD4+CD25+FOXP3– can also represent an effector population (Burl et al., 2010); whilst these were increased over co-stimulation alone by stimulation with EB, the levels were not significantly different between TT cases and unaffected controls. Interestingly, we found that IFNγ produced in response to *C. trachomatis* EB was not exclusive to CD4+ lymphocytes, and the *in vitro* nature of the stimulation with non-viable EBs would not efficiently favor

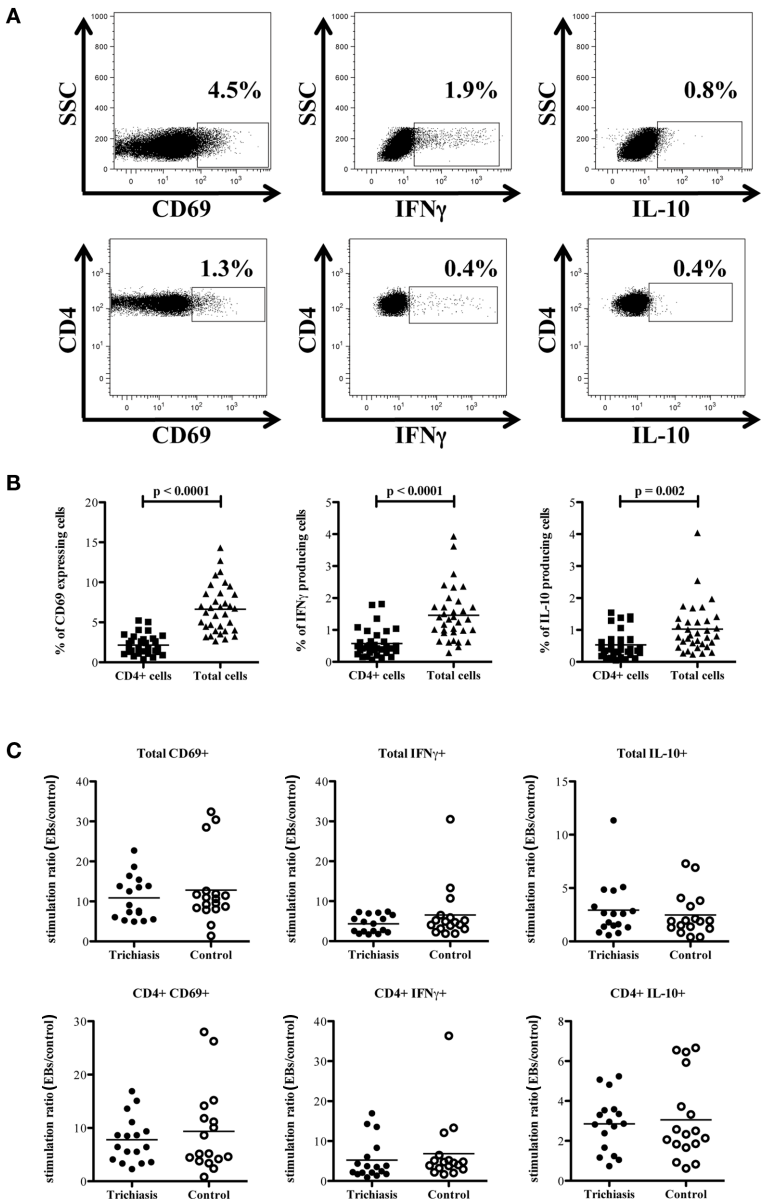

FIGURE 4 | CD4+ cells are not the primary source of IFNγ following stimulation with EBs. **(A)** Representative FACS plots of whole blood cultured for 48 h with EBs and co-stimulatory CD28/CD49d demonstrate the gating strategy used to distinguish between total and CD4+ IFNγ producing cells, as well as total and CD4+ IL-10 producing cells. **(B)** Percentage of total IFNγ+, CD69+, and IL-10 producing cells is significantly higher than the percentage of CD4+ IFNγ+, CD4+CD69+, and CD4+IL-10+ cells in all trichiasis cases and controls ($n = 34$), indicating that CD4+ T cells are not the only source of IFNγ, IL-10, and activated cells. **(C)** Responses from trichiasis cases ($n = 17$) and controls ($n = 17$) represented as a stimulation ratio (as in **Figure 3**). The ratio of CD4+ activated (CD69+) or IFNγ+/IL-10+ cells are slightly higher in the control group but the difference is not significantly different.

stimulation of CD8+ T cells. Given that <50% of the total IFNγ production appeared to be from CD4+ T cells, we investigated using flow cytometry which cells were responsible for the early IFNγ production. We found that even with a restricted sample size and therefore statistical power, CD3−CD56+ NK cells were a major source of very early IFNγ production (within the first 18 h of *in vitro* restimulation). Since the magnitude of this response increased with age and the frequency and duration of trachoma decrease with age (Bailey et al., 1999; Faal et al., 2006), it is possible that these cells play a significant role in ocular disease and infection. Such responses are not unique and are in line with those observed for responses following vaccination or as a result of protozoan or viral infection (Horowitz and Riley, 2010; Horowitz et al., 2010a,b; McCall et al., 2010). Interestingly, in subjects with active trachoma and infection, host gene transcriptional profiles in the conjunctiva suggest a previously unappreciated role for NK cells (Natividad et al., 2010). These initial *in vitro* experiments suggest that NK or NK-like cells are rapidly induced by EB and supports the suggestion that lymphocytes other than CD4+ T cells contribute a significant proportion of IFNγ in the periphery. EB

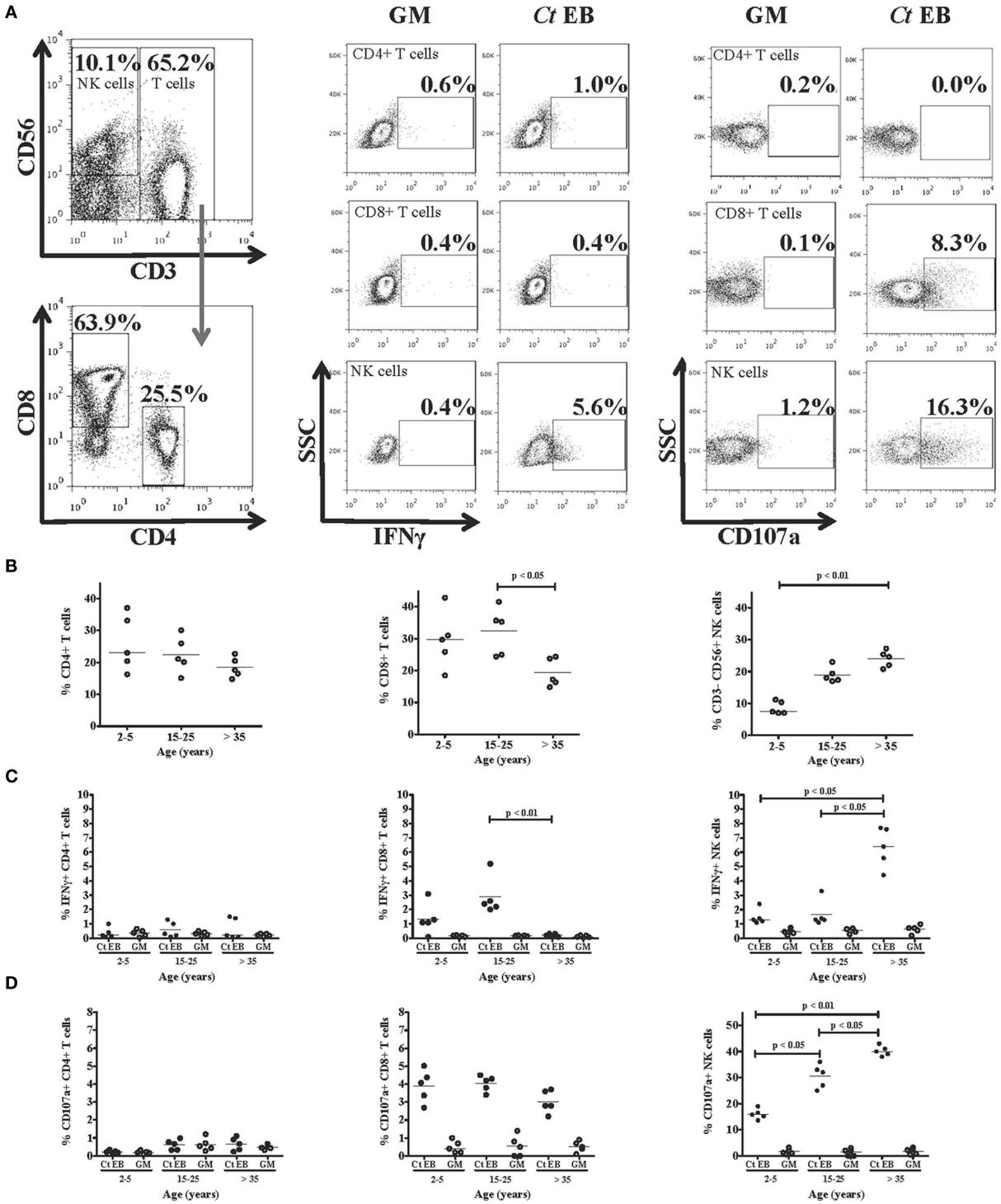

FIGURE 5 | CD3–CD56+ natural killer (NK) cells are a major source of IFNγ in response to EB stimulation that increases with age. PBMC from healthy individuals ranging in age from 2 to 5 years ($n = 5$), 15–25 years ($n = 5$), and >35 years ($n = 5$) were stimulated for 24 h with EB. **(A)** Representative FACS plots demonstrate the gating strategy used to define populations of CD4+ and CD8+T cells and NK cells. NK cells (CD3–CD56+) were gated from CD3+T cells. The CD3+T cells were then further sub-divided based on CD4+ or CD8+ expression. **(B)** Cell percentages in each age group demonstrate the natural variation of CD8+ T and NK cell levels, whereas CD4+ T cell levels do not appear to vary with age. **(C)** The percentage of NK cells producing IFNγ significantly increased with age, while the percentage of CD8+ IFNγ+ cells significantly decreased in the >35 years age group. The percentage of CD4+ IFNγ+ did not differ among the different age groups. **(D)** The percentage of CD107a+ NK cells significantly increased with age, whereas levels of CD107a expression in CD8+T cells did not differ with age. CD4+T cells expressed negligible levels of CD107a.

stimulation also leads to the early activation of other non-CD4+ T cells along with IL-10 production. We did not investigate the phenotypes of the cells that are activated or produce IL-10 and this warrants further more detailed investigation. Some *in vitro* studies have suggested that stimulated T cells rapidly acquire CD56 when strongly stimulated through the CD3/TCR complex with additional IL-2 (Kelly-Rogers et al., 2006) and that the phenotypic profile of NK cells is heterogeneous. Since we stained for both surface and internalized CD3 to identify the classical NK cell population (CD3−CD56+; Meier et al., 2005; Titanji et al., 2008; Li et al., 2009; Linn et al., 2009) we exclude this possibility as an *in vitro* artifact and suggest that the interaction with antigen-experienced T cells should be investigated further in response to chlamydial infection.

The onset of TT usually occurs well into adulthood and previous studies have revealed that *C. trachomatis* is rarely detected in the conjunctiva at that point (Burton et al., 2007). Intriguingly, TT cases continue to progress toward more severe trichiasis and eventually blindness despite their peripheral immune response being comparable to that of individuals with equal exposure to infection but without the clinical signs or sequelae of trachoma. This would suggest that adaptive T cell memory responses in the periphery do not reflect clinical disease status and that other factors such as innate inflammatory responses involving polymorphs (such as neutrophils) and host epithelial cells, continue to drive fibrosis or recurrent trichiasis. This is supported by several previous studies linking conjunctival gene expression and trichiasis (Burton et al., 2010, 2011; Holland et al., 2010). Other factors such as the compromised ocular surface and secondary bacterial infection also contribute to continued inflammation and disease. Thorough sampling of the immune response at the site of infection and a more in-depth examination of the interaction of adaptive T cell responses and NK cell responses may help further probe the mechanisms of immunopathogenesis of trachoma.

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Ribosomal protein S3: a multifunctional target of attaching/effacing bacterial pathogens

Xiaofei Gao and Philip R. Hardwidge*

Department of Microbiology, Molecular Genetics and Immunology, University of Kansas Medical Center, Kansas City, KS, USA

Edited by:
Elizabeth L. Hartland, University of Melbourne, Australia

Reviewed by:
Elizabeth L. Hartland, University of Melbourne, Australia
Toru Tobe, Osaka University, Japan

****Correspondence:***
Philip R. Hardwidge, Department of Microbiology, Molecular Genetics and Immunology, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, KS 66160, USA.
e-mail: phardwidge@kumc.edu

The extraribosomal functions of ribosomal proteins have drawn significant recent attention. Ribosomal protein S3 (RPS3), a component of the eukaryotic 40S ribosomal subunit, is a multifunctional protein that regulates DNA repair, apoptosis, and the innate immune response to bacterial infection. Here we the review the latest findings about RPS3 extraribosomal functions, with special emphasis on their relation to microbial pathogenesis and enteropathogenic *Escherichia coli*.

Keywords: apoptosis, DNA repair, EPEC, extraribosomal function, NF-κB, NleH1, ribosomal protein S3

INTRODUCTION

Ribosomal proteins function not only in protein translation, but also in multiple extraribosomal activities (Blumenthal and Carmichael, 1979). These functions include, but are not limited to, DNA repair, cell death, inflammation, tumorigenesis, and transcriptional regulation (Warner and McIntosh, 2009). Here we focus on a eukaryotic 40S ribosome component, the ribosomal protein S3 (RPS3), and its emerging regulatory roles in DNA repair, apoptosis, and pro-inflammatory signaling during bacterial infection. We propose that RPS3 may play a central role in regulating numerous aspects of host–pathogen interactions.

RPS3 AND MICROBIAL PATHOGENESIS

Ribosomal protein S3 has been directly and indirectly implicated in host–pathogen interactions. A clone of human RPS3 was obtained in a yeast three-hybrid screen designed to identify proteins that bind the 3′ untranslated region (UTR) of hepatitis C virus (Wood et al., 2001). Suppression subtractive hybridization studies of mast cell gene expression modulated by *Pseudomonas aeruginosa* suggested that RPS3 might be involved in *P. aeruginosa* pathogenesis (Sun et al., 2005). RPS3 expression levels may also be important to mouse resistance to the H5N1 influenza virus (Boon et al., 2009).

The NF-κB family of transcription factors regulates the expression of genes involved in a variety of cellular functions such as immune responses and cellular proliferation (Lenardo and Baltimore, 1989). NF-κB is normally sequestered in the cytoplasm by inhibitory IκB proteins that mask NF-κB nuclear localization signals (Hacker and Karin, 2006). After a cell recognizes a pathogen-associated molecular pattern (PAMP), the IκB kinase (IKK) complex is activated and subsequently phosphorylates the IκBs, leading to their ubiquitination and degradation by the 26S proteasome, permitting NF-κB subunits to translocate into the nucleus to function in transcription.

It was recently discovered that RPS3 is also inducibly associated with and phosphorylated by IKKβ on serine 209 (S209) in response to NF-κB pathway activation (Wan et al., 2011). This phosphorylation event is essential to the nuclear translocation of RPS3, after it associates with importin-α (Wan et al., 2011). Affinity purification experiments had also revealed that RPS3 interacts with the p65 NF-κB subunit through its K homology (KH) domain (Wan et al., 2007; **Figure 1**).

After entering the nucleus, NF-κB binds to κB sites within target gene promoters and regulate transcription by recruiting co-activators/repressors (Wan et al., 2007). This newly discovered NF-κB subunit, RPS3, guides NF-κB to specific κB sites by increasing the affinity of the p65 NF-κB subunit for a subset of target gene promoters (Wan et al., 2007). Likewise, silencing RPS3 expression alters a subset of NF-κB signal transduction pathways. RPS3 thus provides for selective NF-κB recruitment to specific promoters and tailors cellular transcriptional responses to specific stimuli. Interestingly, RPS3 also forms a complex with NF-κB in human islet cells after stimulation with IL-1β (Mokhtari et al., 2009).

The function of type III secretion system (T3SS) effector proteins has been a subject of intense research in recent years (Dean and Kenny, 2009). Some effectors (e.g., NleB, NleC, NleD, NleE, NleH) are key modulators of the innate immune system of intestinal epithelial cells, especially pathways regulated by NF-κB. For example, NleC is a protease that cleaves the NF-κB p65 subunit (Marches et al., 2005; Yen et al., 2010; Baruch et al., 2011; Muhlen et al., 2011; Pearson et al., 2011). NleD cleaves the c-Jun N-terminal kinase (JNK) thus blocking activator protein-1 (AP-1) activation (Baruch et al., 2011). NleE inhibits both p65 nuclear translocation and IκBα degradation (Newton et al., 2010) to block NF-κB activation, in conjunction with NleB (Nadler et al., 2010; Newton et al., 2010).

During attaching/effacing (A/E) pathogen infection, the T3SS effectors NleH1 and NleH2 bind to the N-terminus of RPS3 after their translocation into host cells (Gao et al., 2009). NleH1, but not NleH2, inhibits the nuclear translocation of RPS3, consequently inhibiting the transcription of genes encoding pro-inflammatory

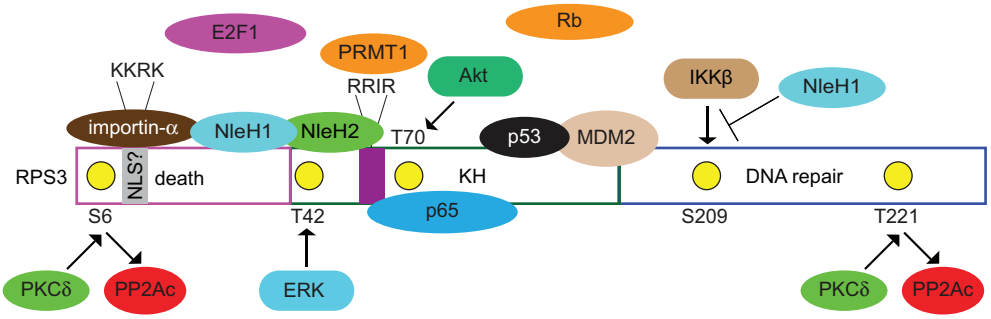

FIGURE 1 | Known and postulated interactions between RPS3 and mammalian or bacterial proteins. Specific RPS3 phosphorylation sites and the protein kinases responsible (PKCδ, ERK, Akt) are indicated. NleH1 and NleH2 are *E. coli* virulence proteins. NleH1 inhibits the IKKβ-mediated phosphorylation of RSP3 S209. Figure is not drawn to scale and specific protein–protein interactions and/or binding interfaces should not be inferred, except where specifically indicated in the text.

cytokines, such as IL-8 and TNF-α, indicating that pathogens target RPS3 to inhibit host immune defenses (Gao et al., 2009).

NleH1 functions by inhibiting the IKKβ-mediated phosphorylation of RPS3 S209 (Wan et al., 2011). NleH1 is an autophosphorylated Ser/Thr protein kinase with an active site at lysine 159 (K159; Gao et al., 2009). While the kinase substrate for NleH1 is not yet known, it does not appear to phosphorylate either IKKβ or RPS3. However, NleH1 kinase activity is required to inhibit IKKβ from phosphorylating RPS3, as mutating the NleH1 K159 residue to alanine (K159A) prevented NleH1 from inhibiting RPS3 S209 phosphorylation, both *in vitro* and in cell culture models (Wan et al., 2011). Studies of gnotobiotic piglets infected with *Escherichia coli* O157:H7 also demonstrated that RPS3 S209 phosphorylation is inhibited by NleH1 *in vivo*, possibly to benefit bacterial colonization and transmission (Wan et al., 2011).

It is interesting that IKKβ activation and IκBα degradation appear to be unaffected by NleH1 (Wan et al., 2011) suggesting that it may be beneficial for the pathogen to attenuate the transcription of RPS3-dependent, but not all NF-κB-dependent target genes. It will be important to consider how the apparently selective alteration of NF-κB activity achieved by NleH1 is coordinated with the other enteropathogenic *E. coli* (EPEC) effectors targeting the NF-κB pathway.

NleH also functions in preventing host cell apoptosis through a mechanism likely to be independent of its interaction with RPS3 (the role of RPS3 in regulating apoptosis will be discussed below). The EPEC effector EspF disrupts host mitochondrial membrane potential and induces the degradation of the anti-apoptotic protein Abcf2 (Nougayrede et al., 2007). Despite the pro-apoptotic function of EspF, EPEC does not induce a large degree of apoptosis, suggesting that other effectors may have anti-apoptotic function. Indeed, an EPEC mutant deleted for both *nleH1* and *nleH2* reduced host cell survival as compared with wild-type EPEC infection (Hemrajani et al., 2010).

Yeast two-hybrid studies subsequently revealed that NleH1 binds to the Bax inhibitor-1 (BI-1) protein (Hemrajani et al., 2010). This result is interesting because the intrinsic pro-apoptotic pathway involves the activation of Bcl-2-homology 3-only (BH3) proteins, as well as the oligomerization of Bak/Bax proteins. Transfecting NleH1 prevented caspase-3 activation (Hemrajani et al., 2010), as well as *Clostridium* TcdB-induced apoptosis (Robinson et al., 2010). Interestingly, a pro-apoptotic Bcl-2 protein, the BH3 interacting domain death agonist (BID) was recently shown to interact with the nucleotide-binding oligomerization domain-containing proteins NOD1 and NOD2, as well as the IKK complex, thus integrating apoptosis and NF-κB signaling (Yeretssian et al., 2011).

NleH1 also interacts with the Na^+/H^+-exchange regulatory factor 2 (NHERF2) at the plasma membrane (Martinez et al., 2010). Because over-expressing NHERF2 reduces the anti-apoptotic function of NleH1, it has been suggested that NHERF2 may serve as a plasma membrane sorting site to bind bacterial effector proteins away from other host targets (Martinez et al., 2010).

DNA REPAIR

It was determined, after the *Drosophila melanogaster* RPS3 cDNA was cloned (Wilson et al., 1993), that RPS3 cleaves DNA at apurinic/apyrimidinic (AP) sites of DNA damage (Wilson et al., 1993). The AP site is a DNA lesion which, without removal, can halt mRNA and DNA synthesis and cause cell death (Loeb and Preston, 1986). *Drosophila* RPS3 possesses an N-glycosylase activity and liberates 8-oxoguanine (8-oxoG) DNA lesions generated during oxidative stress (Yacoub et al., 1996; Deutsch et al., 1997). Transforming RPS3 into *E. coli* rescues the H_2O_2 sensitivity of an *E. coli mutM* strain, as well as the alkylation sensitivity of exo III and endo IV *E. coli* mutants (Yacoub et al., 1996). *Drosophila* RPS3 also accelerates the repair of 8-oxoG lesions in both human and mouse cell extracts (Cappelli et al., 2003).

Both human (Hegde et al., 2004a) and yeast (Jung et al., 2001) RPS3 are also involved in DNA repair. Over-expressing yeast RPS3 overcomes both the osmotic and oxidative stress sensitivity normally observed in a *yar1* mutant, a gene encoding an ankyrin-rich repeat protein that serves a stress response function (Loar et al., 2004). The RPS3 gene is found in a single copy in *Saccharomyces cerevisiae* and its disruption yields non-viable haploid spores (Fingen-Eigen et al., 1996). Human RPS3 binds tightly to both AP and 8-oxoG sites (Hegde et al., 2004b), but appears not to possess its own glycosylase activity (Kim et al., 2005a). Human RPS3 instead functions by binding to and stimulating the activities

of a uracil-DNA glycosylase (Ko et al., 2008), as well as the base excision repair (BER) enzymes hOGG1 and APE/Ref-1 (Hegde et al., 2004a), both of which are multifunctional proteins with AP endonuclease activity.

Ribosomal protein S3 nuclear translocation and its subsequent participation in DNA repair are governed by several post-translational modifications. While RPS3 contains a putative nuclear localization sequence (AAs 7–10; KKRK), no data are yet available to indicate definitively whether this sequence motif is essential to RPS3 nuclear translocation. RPS3 nuclear translocation in response to DNA damage (Yadavilli et al., 2007) is regulated by the extracellular signal-regulated kinase 1 (ERK1), which phosphorylates RPS3 on T42 (Kim et al., 2005b; **Figure 2**). This phosphorylation is critical to regulating RPS3 nuclear translocation, as an RPS3 T42A mutant is significantly reduced in nuclear abundance, even after extensive DNA damage is induced by H_2O_2 treatment. In contrast, an RPS3 T42D mutant is constitutively localized to the nucleus, even in the absence of DNA damage (Yadavilli et al., 2007).

Protein kinase C delta (PKCδ) is a serine–threonine protein kinase that can function in cellular responses relating to DNA damage (Yoshida, 2007). PKCδ phosphorylates the S6 and T221 residues of RPS3 (Kim et al., 2009a). Chemicals that activate PKCδ [e.g., phorbol myristate acetate (PMA) or H_2O_2] increase both the extent of phosphorylation and the repair endonuclease activity of RPS3 (Kim et al., 2009a). After nuclear translocation, the N-terminus of RPS3 can be bound by the catalytic subunit of protein phosphatase 2A (PP2Ac; Kim et al., 2009b) and it appears that prior phosphorylation of RPS3 on S6/T221 is necessary for this interaction (Kim et al., 2009b).

A recent study showed that EPEC enters crypts of the human colon (Maddocks et al., 2009). Studies with human colorectal cell cultures demonstrated that EPEC downregulates the expression of the mismatch repair proteins MLH1 and MSH2, in a mechanism independent of apoptosis (Maddocks et al., 2009). It will be interesting to determine the extent to which EPEC effector protein interactions with RPS3 might account for this phenotype.

APOPTOSIS

Several ribosomal proteins, including RPS3, regulate apoptosis (Naora et al., 1998; Khanna et al., 2003; He and Sun, 2007). Mutational analysis of RPS3 suggests that RPS3 amino acids 15–26,

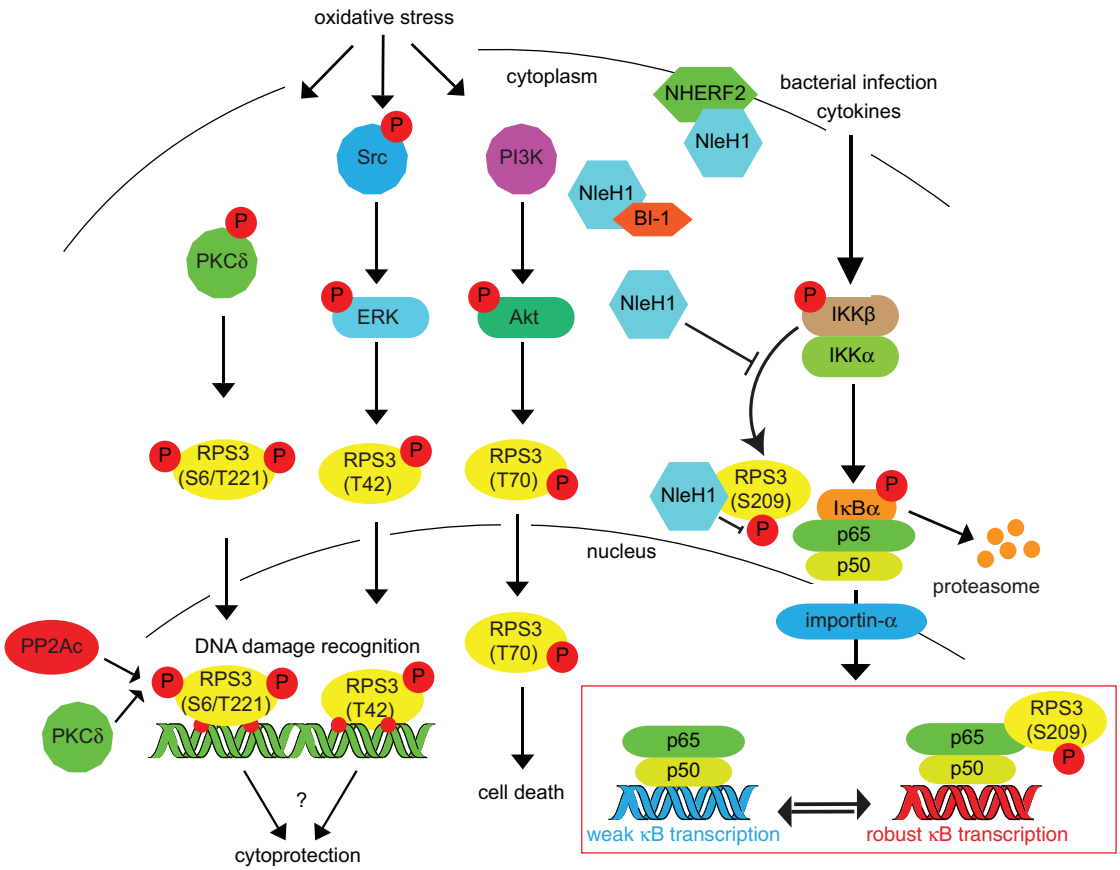

FIGURE 2 | Inducing RPS3 phosphorylation via oxidative stress, DNA damage, and bacterial infection. RPS3 phosphorylation is activated by a variety of cellular stressors. The specific phosphorylation sites and the protein kinases responsible (PKCδ, ERK, Akt) are indicated. Nuclear RPS3 is also bound by the catalytic subunit of protein phosphatase 2A (PP2Ac). IKKβ phosphorylates RPS3 S209 in response to cytokines or bacterial infection. This phosphorylation can be inhibited by the *E. coli* NleH1 protein, which also binds the BI-1 and NHERF2 proteins.

the "death domain," are critical to the function of RPS3 in inducing apoptosis (Jang et al., 2004). Over-expressing an RPS3–GFP fusion induces DNA condensation and promotes the degradation of both the poly (ADP-ribose) polymerase (PARP) and lamin A/C (Jang et al., 2004; Lee et al., 2010), both of which are hallmarks of apoptotic induction. By contrast, depleting endogenous RPS3 rescues cell survival under oxidative stress conditions (Hegde et al., 2007). Caspase-3, -8, and -9 are activated by over-expressing RPS3 in mouse MPC-11 cells, indicating that RPS3-induced apoptosis is likely to be caspase-dependent (Jang et al., 2004).

In addition, either over-expressing or knocking down RPS3 expression levels can lead to apoptosis, suggesting that the total abundance of RPS3 is important to proper cellular function. Mouse embryonic fibroblasts (MEFs) derived from mice engineered to over-express RPS3 display increased levels of DNA damage after oxidative stress, possibly attributable to RPS3 binding to 8-oxoG and blocking BER activities (Hegde et al., 2009). However, in other systems, in the presence of DNA damaging agents (e.g., H_2O_2 and methyl methanesulfonate; MMS), knocking down RPS3 actually leads to increased cell survival, by relieving the RPS3-obstacle to liberating 8-oxoG from damaged DNA (Hegde et al., 2007). Knocking down *C. elegans* RPS3 expression after worms reach adulthood increases lifespan (Curran and Ruvkun, 2007), but the mechanism was not studied in detail.

Ribosomal protein S3 is a substrate for another kinase, Akt (Lee et al., 2010), which can be activated by insulin and pro-survival factors. Activated Akt phosphorylates a variety of cellular proteins involved in the cell cycle, cell survival, and metabolism. Akt phosphorylates the RPS3 T70 residue, also promoting RPS3 nucleus translocation. In this case, T70 phosphorylation is suggested to prevent RPS3-induced apoptosis (Lee et al., 2010). In neuronal cells, RPS3 induces apoptosis by upregulating the expression of pro-apoptotic BH3-only proteins such as Bim and the activator of apoptosis harakiri (Dp5/HRK) by interacting with transcription factor E2F1 (Hershko and Ginsberg, 2004; Lee et al., 2010). Akt-dependent phosphorylation of RPS3 T70 blocks the pro-apoptotic function of RPS3 by inhibiting its interaction with E2F1 while concomitantly enhancing RPS3 endonuclease activity (Lee et al., 2010). These authors also indicate (Lee et al., 2010), in unpublished data, that RPS3 binds to the retinoblastoma (Rb) protein.

A model for RPS3-induced apoptosis has been proposed. Under oxidative stress, RPS3 is phosphorylated by host protein kinases, including ERK, PKCδ, and Akt, consequently translocating into the nucleus to undertake its DNA repair function. Excessive BER may cause irreversible DNA damage and lead to apoptosis (Jang et al., 2004). Thus, increased RPS3 activity at sites of damaged DNA could lead RPS3 to function as an apoptosis signal mediator through a DNA repair enzyme. It is not yet clear what is the mechanism governing cellular apoptosis induced by RPS3 knockdown, though some have speculated that it is related to disrupting protein translation (Lee et al., 2010).

CANCER AND p53

Many studies have identified transcripts and proteins that are differentially expressed in cancer. These proteins include many ribosomal proteins and, among them, RPS3. RPS3 expression is increased in adenocarcinomas and in the majority of adenomatous polyps (Pogue-Geile et al., 1991). Suppression subtractive hybridization also identified RPS3 as over-expressed in a leukemia cell line (Zhu et al., 2003). In contrast, RPS3 appears to be under-expressed in squamous cell lung carcinomas (McDoniels-Silvers et al., 2002).

Ribosomal proteins regulate p53 activity, a tumor suppressor involved in arresting the cell cycle and inducing apoptosis (Sulic et al., 2005; Panic et al., 2006; Chakraborty et al., 2009). Zebrafish with heterozygous mutations in genes encoding 17 different ribosomal proteins are impaired in p53 protein production and develop a rare malignant peripheral nerve sheath tumor (Amsterdam et al., 2004). Disrupting the regulation of p53 protein production may lead to tumorigenesis (MacInnes et al., 2008). p53 Levels are normally regulated by MDM2, which possesses an E3 ubiquitin ligase activity that promotes p53 degradation (Honda et al., 1997). However, during nucleolar stress, ribosomal proteins interact with the acidic zinc finger region of MDM2 (Horn and Vousden, 2008), limiting the function of MDM2.

Ribosomal protein S3 interacts, via its KH RNA-binding domain, with both MDM2 and p53 (Yadavilli et al., 2009). p53 and MDM2 levels normally increase and decrease, respectively, after oxidative stress. By contrast, in cells knocked down for RPS3 expression, p53 levels decrease and the E3 ligase activity of MDM2 is lost (Yadavilli et al., 2009), suggesting RPS3 is important to stabilizing p53.

Ribosomal protein S3 can also interact, via its N-terminus, with a nucleoside diphophate kinase, NM23-H1, that is activated during apoptosis and may function as a tumor suppressor (Kim and Kim, 2006). Over-expressing RPS3 reduces invasion by human fibrosarcoma cells and reduced matrix metalloproteinase 9 (MMP-9) secretion and ERK activation in HT1080 cells (Kim and Kim, 2006). NM23-H1 supresses tumor invasiveness by attenuating Ras-Raf-MEK-ERK signaling (Hartsough et al., 2002). As MMP secretion is regulated by ERK and is thought to be critical to tumor cell invasiveness (Lakka et al., 2002), RPS3 interaction with both NM23-H1 and ERK may play an important role in antagonizing cancer development.

SUMMARY

Ribosomal protein S3 is involved in a broad range of physiological activities. It is reasonable to speculate that interrupting ribosomal protein function via environmental stress or infection will lead to changes in host cell survival. Future studies are likely to reveal additional surprises about the extraribosomal functions of ribosomal proteins and the extent to which these functions are targeted for subversion by the T3SS effectors of A/E pathogens.

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A functional slow recycling pathway of transferrin is required for growth of *Chlamydia*

Scot P. Ouellette*† *and Rey A. Carabeo*

Centre for Molecular Microbiology and Infection, Division of Cell and Molecular Biology, Imperial College London, London, UK

Edited by:
D. Scott Merrell, Uniformed Services University, USA

Reviewed by:
Luiz Bermudez, Oregon State University, USA
Daniel E. Voth, University of Arkansas for Medical Sciences, USA

****Correspondence:***
Rey A. Carabeo, Centre for Molecular Microbiology and Infection, Flowers Building, Imperial College London, London SW7 2AZ, UK.
e-mail: r.carabeo@imperial.ac.uk

*†**Current address:***
Scot P. Ouellette, Unite de Biochimie et Interactions Macromoleculaires, Institut Pasteur, 75724 Paris, France.
e-mail: scot.ouellette@pasteur.fr

An inhibitor of host cell lysophospholipid acyltransferase, an enzyme involved in lipid metabolism blocked growth of the obligate intracellular pathogen *Chlamydia* through its action on the transport of transferrin (Tf) via the slow pathway of recycling. A detailed characterization of this inhibition revealed that Tf accumulated in vesicles positive for Rab11, with a concomitant reduction in the level of Tf found within the transport intermediate Rab4/11 hybrid vesicles. The net result was the failure to be recycled to the plasma membrane. In chlamydiae-infected cells, the Tf-containing Rab11-positive vesicles were typically found intimately associated with the inclusion, and treatment with the inhibitor caused their accumulation, suggesting that the timely progression and completion of Tf recycling was necessary for proper chlamydial growth. Growth inhibition by the compound could be negated by the simple removal of the Tf-containing fraction of the serum, a further indication that accumulation of Tf around the chlamydial inclusion was deleterious to the pathogen. Thus, it appears that manipulating the slow recycling pathway can have biological consequences for *Chlamydia* and implies the need to regulate carefully the interaction of the inclusion with this host trafficking pathway.

Keywords: *Chlamydia*, inclusion, slow transferrin recycling pathway, LPAT, iron

INTRODUCTION

Chlamydia is a developmentally regulated bacterium that resides within its target host cell in a pathogen-defined protective niche called an inclusion (Hackstadt, 2000). Chlamydiae do not acquire markers of the endocytic pathway but rather appear to intercept exocytic traffic as demonstrated by the acquisition of sphingomyelin (SM) en route from the trans-Golgi network to the plasma membrane (Hackstadt et al., 1996). Furthermore, the chlamydial inclusion displays selectivity in its interactions with the exocytic pathway by excluding vesicles containing glycoprotein cargo (Scidmore et al., 1996a). The significance of SM transport to the inclusion is unclear although a recent report by Heuer et al. (2009) demonstrated the role of infection-induced Golgi fragmentation in chlamydial growth, which correlated the fragmentation of the Golgi apparatus with enhanced SM transport to the inclusion. However, inhibiting this process had only modest effects on chlamydial growth.

Recent reports from a number of laboratories have begun to challenge the limited fusogenicity of the inclusion with vesicular transport pathways. Several Rab GTPases, including Rab4 and Rab11, have been localized to the inclusion membrane, and chlamydial proteins that interact with them have been identified (Rzomp et al., 2003, 2006; Cortes et al., 2007). In addition, lipid droplets have been found inside chlamydial inclusions (Cocchiaro et al., 2008), thus the repertoire of organelles and vesicles with which *Chlamydia* interacts is likely to grow.

The observation that Tf-containing vesicles localized around the inclusion throughout the chlamydial developmental cycle has been repeatedly confirmed (Scidmore et al., 1996b, 2003; van Ooij et al., 1997; Al-Younes et al., 1999; Rzomp et al., 2003). However, the significance to *Chlamydia* of this association has not been elucidated. Expression of dominant-negative mutants and knockdown by RNA interference to interfere with Rab4 function failed to reveal any role in chlamydial growth. Recently, the Rab11 GTPase, which functions in the slow pathway of Tf recycling, was implicated in chlamydial growth but at the level of the Golgi-inclusion interaction (Rejman Lipinski et al., 2009). Without any data that correlates inhibition of the transferrin (Tf) recycling pathway with abrogation of chlamydial growth, the significance of the localization of these vesicles relative to the inclusion remains uncertain.

Transferrin recycling could be divided into two pathways based on the kinetics of recycling to the plasma membrane. Approximately 80–90% of internalized Tf are recycled through the fast/bulk pathway within 30 min after internalization; and the recycling Tf transits through vesicular compartments positive for Rab5 and/or Rab4 GTPases. The remaining population of Tf is retained much longer in Rab4 and/or Rab11 positive vesicles within the endocytic recycling compartment, and has a longer duration (60–90 min) of recycling (Sönnichsen et al., 2000). Given this added complexity of Tf recycling, we sought to examine the involvement of both pathways in chlamydial growth.

Here, we describe the characterization of a potent inhibitor of Tf recycling with regards to its effect on chlamydial growth. A screen of a small molecule library identified this compound as a potent inhibitor of chlamydial growth. The inhibitor (FR179254) targeted both the enzyme acyl-CoA:cholesterol acyltransferase (ACAT) and lysophospholipid acyltransferase (LPAT). The demonstrated lack of anti-chlamydial activity of an ACAT-specific inhibitor (Sandoz 58-035) or the normal growth of chlamydia in ACAT-deficient cell lines indicated that the relevant activity was

indeed LPAT. This was confirmed by the use of the CI-976 compound, which has been characterized to specifically inhibit LPAT (Chambers and Brown, 2004). FR179254 targeted the transport of Tf through the Rab4/Rab11 double-positive vesicle, which is an intermediate transport compartment of the slow recycling pathway. The net result was the accumulation of Tf in Rab11-positive vesicles. This inhibitor-induced Tf accumulation was observed in both infected and uninfected cells. Interestingly, we found that the removal of Tf-containing fractions from the serum in an attempt to prevent accumulation rendered FR179254 ineffective in inhibiting chlamydial growth thus linking abnormal Tf recycling with chlamydia development. Furthermore, both Tf accumulation and inhibition of chlamydial growth could be mimicked partially by overexpressing the dominant-negative mutants of Rab4 and Rab11, confirming a role for these Rab GTPases in this process. The observed biological link between Tf-containing vesicles and the chlamydial inclusion reveals a different picture of the inclusion – a pathogen-defined organelle that is highly interactive with host vesicular traffic.

MATERIALS AND METHODS

ORGANISMS AND CELL CULTURE

Chlamydia trachomatis L2 EBs were harvested from infected HEp-2 or HeLa cell cultures at 37°C with 5% CO_2, purified by discontinuous density gradient centrifugation in Renografin (Bracco Diagnostics, Princeton, NJ, USA), and titered for infectivity as measured by inclusion forming units (IFU). *Escherichia coli* MG1655 and the temperature-sensitive Δ*plsC* (Coli Genetic Stock Center, Yale University, New Haven, CT, USA) bacteria were cultured on LB plates or broth using standard bacterial culture protocols with the mutant being routinely cultured at 30°C. HEp-2, HeLa, COS7, CHO, or AC29 (a kind gift of Dr. Chang, Dartmouth University) cells were routinely cultivated at 37°C with 5% CO_2 in IMDM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 10 μg/mL gentamicin (all from Invitrogen, Carlsbad, CA, USA). For some experiments, IMDM lacking serum (iIMDM) was used in the presence or absence of FR179254 (Calbiochem, La Jolla, CA, USA). Serum fractionations were performed sequentially using 50 or 100 kDa cut-off filters (Millipore) to obtain three fractions representing less than 50 kDa, 50–100 kDa, and greater than 100 kDa. Fractions were analyzed by standard SDS-PAGE techniques and Western blot using a rabbit polyclonal anti-Tf antibody (Abcam, Cambridge, UK), and compared to total serum and purified holo-Tf (Sigma, St. Louis, MO, USA). Each fraction was normalized for volume and subsequently added to iIMDM to a final concentration of 10%.

QUANTIFICATION OF IFUs FROM INFECTED CELL CULTURES

HEp-2 cells were plated in 6-well culture plates at a density of 10^6 cells per well. In a subset of wells, cells were plated onto glass cover slips for immunofluorescence microscopy. Approximately 18 h later, cells were infected at an MOI of 1 by direct addition to each well and left untreated or treated with 20 μM FR179254, 20 μM U18666a (Calbiochem, La Jolla, CA, USA), 5 μM Triacsin C, 50 μM CI-976, or 5 μg/mL Sandoz 58-035 (all from Sigma, St. Louis, MO, USA) and infected cells were incubated at 37°C with 5% CO_2. For oleic acid experiments, cells were treated with 300 μM oleic acid (Sigma) at the time of plating or during reactivation as indicated. For recovery experiments, cells were cultured in serum-free IMDM or IMDM supplemented with serum fractions in the presence and absence of FR179254. For all samples, addition of inoculum to wells marks the time of infection ($t = 0$ h). Medium was aspirated from the treatment samples of infected cells at 24 h p.i., and 1 mL of SPG was added to each well. Cells were scraped and collected from each well into a 1.5-mL microfuge tube with three glass beads. Samples were vortexed for 45 s and frozen at −80°C. Samples were titered for infectivity on fresh cell layers as described elsewhere to quantify the number of IFU per well (Ouellette et al., 2006). At 24 h p.i., cells plated on cover slips were washed with PBS, fixed with methanol for 10 min, and carefully washed three times with PBS plus 0.025% sodium azide. Infected cultures were stained with primary human polyclonal antibody (Ab) against chlamydiae and secondary Ab goat anti-human Alexa488 (Invitrogen). Immunofluorescence was viewed on an Olympus Fluoview 500 Laser Scanning Microscope.

FLUORESCENT MICROSCOPY

For all of these assays, HEp-2 cells were plated at 10^5 cells per well on 10 mm cover slips in 24-well tissue culture plates. Cells were processed as described after 24 h infection and/or treatment with drugs and visualized by confocal microscopy except where indicated. For visualization of lipid droplets, live cells were incubated in the presence of 1 μg/mL Bodipy 493/503 (Invitrogen) under normal culture conditions for approximately 10 min, rinsed 3× with HBSS, and viewed live on a Zeiss Axio Imager.M1 deconvolution microscope. For fluid-phase uptake, live cells were incubated in 50 μg/mL dextran-647 (10000 MW, fixable; Invitrogen) for 2 h, rinsed 3× with HBSS, fixed in paraformaldehyde, and imaged. All images were acquired using the same exposure settings on the microscope. CD63-GFP cells were a kind gift of Dr. Frank Dorsey (Scripps FL). Cells were infected and treated as indicated and viewed live 24 h p.i. The GFP signal was verified to co-localize with a CD63 Ab (data not shown). For SM labeling, control or pre-treated cells were pulse-labeled with 5 μM BodipyFL-ceramide complexed with defatted BSA (dfBSA) (Invitrogen) diluted in IMDM without supplements for 30 min at 4°C, washed 3× with HBSS, and chased for 1 h at 37°C in back-extraction medium (IMDM + 1% dfBSA) with or without drugs as indicated. Cells were then imaged live to visualize inclusions and Golgi. For retrograde trafficking, control cells or cells pre-treated with drugs were labeled with 10 μg/mL of Alexa488 labeled cholera toxin subunit B (CTxB-488; Invitrogen) diluted in IMDM medium without any supplements. CTxB-488 was added to cells for 1 h at 4°C to pulse-label cell membranes, washed 3× with HBSS, and chased by incubating for 1 h at 37°C in medium with or without drugs as indicated. Separate samples were fixed in paraformaldehyde after the pulse and chase and imaged.

TRANSFECTIONS

PCR-amplified LAMP-1 sequences were inserted into pEYFP-N1 (CLONTECH Laboratories, Mountain View, CA, USA) to create YFP–LAMP-1 (from Dr. Joel Swanson: Henry et al., 2004). Human cDNA to CD4 cloned into pCMV6-XL5 was obtained from Origene (Rockville, MD, USA). Rab4-GFP (wild-type and dominant-negative) was a kind gift of Dr. Marci Scidmore (Cornell

University: Rzomp et al., 2006). The 2x-FYVE-GFP construct was a kind gift of Dr. Jean Celli (Rocky Mountain Labs, NIAID). Rab11 and Rab5 constructs were obtained from Addgene. One microgram of plasmid was transfected into cells using Fugene 6 (Roche, Indianapolis, IN, USA) according to the manufacturer's directions. Briefly, control or pre-treated cells were incubated in Opti-MEM (Invitrogen). One microgram of plasmid DNA was mixed with Fugene 6 reagent diluted in Opti-MEM for 30 min and then added to cells for 4 h. Medium with or without drugs was added to cells, and cultures were incubated overnight. The following day, cells were fixed in paraformaldehyde, in some cases after a pulse with fluorescently tagged Tf and chased as described. In most cases, cells were visualized directly. For CD4, non-permeabilized cells were incubated with primary Ab mouse anti-human CD4 (R&D Systems, Minneapolis, MN, USA) followed by secondary goat anti-mouse Alexa488 (Invitrogen). The antibody stained cells were then visualized. For the dominant-negative studies, inclusion sizes within infected cells were quantified using ImageJ software (NIH, Bethesda, MD, USA).

TRANSFERRIN RECYCLING

Transferrin conjugated to Alexa488 or Alexa647 (Tf488 or Tf647, respectively; Invitrogen) was diluted to 50 μg/mL in IMDM without supplements. Control or pre-treated cells were pulse-labeled with 250 μL of Tf488/Tf647 medium for 1 h at 4°C, washed 3× with HBSS, and chased by incubating at 37°C in medium with or without drugs. At the indicated times, cells were fixed with 4% paraformaldehyde overnight at 4°C and viewed by confocal microscopy. Images were taken from multiple fields of view across the cover slip using the same exposure settings on the microscope and analyzed for total fluorescence intensity within the field of view using ImageJ software.

ASSAY FOR INHIBITION OF plsC

The *plsC* mutant phenotype has been characterized by Coleman as showing a growth defect at 42°C (Coleman, 1990). Wild-type *E. coli* MG1655 or the Δ*plsC* strain from a single colony were grown overnight at 37°C or 30°C in 5 mL LB broth, respectively. The following day, bacteria were quantified by OD_{600} and diluted in 5 mL LB broth to normalize bacterial numbers. Cultures were grown for 4 h to ensure mid-log phase growth and then sub-cultured again (after normalizing bacterial numbers) in 5 mL broth in the presence of 100 μM FR179254 or CI-976, or 50 μg/mL 58-035, or 34 μg/mL chloramphenicol, or 0.5% DMSO and grown for up to 8 h at either 37°C/30°C or 42°C. OD600 readings were taken to quantify bacterial growth.

RESULTS

FR179254 BLOCKS CHLAMYDIAL GROWTH

We screened a library of low-molecular weight compounds for their ability to abolish chlamydial growth. One of the compounds identified from the screen was a known inhibitor of the host ACAT, FR179254 (Tanaka et al., 1998). A more detailed characterization of its anti-chlamydial activity showed FR179254 at 20 μM to dramatically inhibit growth of the rapidly growing *C. trachomatis* serovar L2, resulting smaller and less numerous inclusions and lower yields of IFU assay (**Figure 1A**). In contrast, the unrelated compound U18666a, which affects a number of transport pathways due to its effects on cellular cholesterol homeostasis (Higgins et al., 1999), did not have observable effects on the inclusion phenotype and only minimal effects on IFU yield. This indicated that the inhibition of chlamydial growth may be specific to FR179254. The activity of FR179254 was verified biochemically by monitoring the levels of unesterified cholesterol that would result from inhibition of the ACAT enzyme by the compound. U18666A did not show inhibitory activity toward cholesterol ester synthesis. In testing other ACAT inhibitors, we found the structurally unrelated CI-976, which has been reported to inhibit the host cell LPAT (Chambers and Brown, 2004), to also block chlamydial growth to a similar extent as FR179254 (**Figure 1A**). We concluded, therefore, that FR179254, like CI-976, is blocking chlamydial growth through its inhibitory action on LPAT.

FR179254 ABROGATES CHLAMYDIAL GROWTH INDEPENDENTLY OF ACAT ACTIVITY

We wanted to further exclude host cell ACAT activity in chlamydial growth. To examine this, we used two approaches – comparing different compounds with established specificity and the use of a mutant Chinese hamster ovary (CHO) cell derivative that lacks significant ACAT activity (AC29) (Cadigan et al., 1988). Firstly, using wild-type CHO cells, we monitored chlamydial growth in cells treated with a specific ACAT inhibitor, Sandoz 58-035. This compound had no effect on chlamydial growth in contrast to FR179254 (**Figure 1B**). Secondly, the CHO mutant AC29 cells remained competent in supporting chlamydial growth. As with CHO cells, 58-035 had no effect on chlamydial growth in AC29 cells whereas FR179254 severely blocked growth in an ACAT-deficient background (**Figure 1B**). These data give further support to LPAT, and not ACAT, as the target of FR179254.

PROKARYOTIC LPAT-LIKE ENZYMES ARE NOT A TARGET FOR FR179254

We wanted to disprove that chlamydiae were the target of the LPAT inhibitors. This is particularly important because chlamydiae have a predicted LPAT-like activity. In *E. coli* the homologous gene, which encodes the LPAT-like activity, is *plsC* (Zhang and Rock, 2008), and mutants of *plsC* show a growth defect at 42°C (Coleman, 1990). Because of the genetic intractability of chlamydiae, we took advantage of the E. coli surrogate experimental system, and assayed the effects of our various inhibitors during growth at both 37°C and 42°C, with the latter mimicking the temperature-sensitive phenotype of the Δ*plsC* mutant. We reasoned that if any of our inhibitors were targeting PlsC, then growth should be abrogated at 42°C. We cultured *E. coli* in the presence of 100 μM inhibitor (5× the concentration used to inhibit *Chlamydia in vitro*) or chloramphenicol as a negative control. As a reference, we also monitored the growth of the reference Δ*plsC* mutant at both temperatures. None of the inhibitors we tested, aside from chloramphenicol, was effective at blocking *E. coli* growth at either temperature as judged by OD readings and colony forming units (**Figure 1C**; data not shown). The Δ*plsC* mutant strain showed the expected growth inhibitory phenotype at the non-permissive temperature (**Figure 1D**). Consequently, it is unlikely that CI-976 or FR179254 are targeting the prokaryotic LPAT homolog.

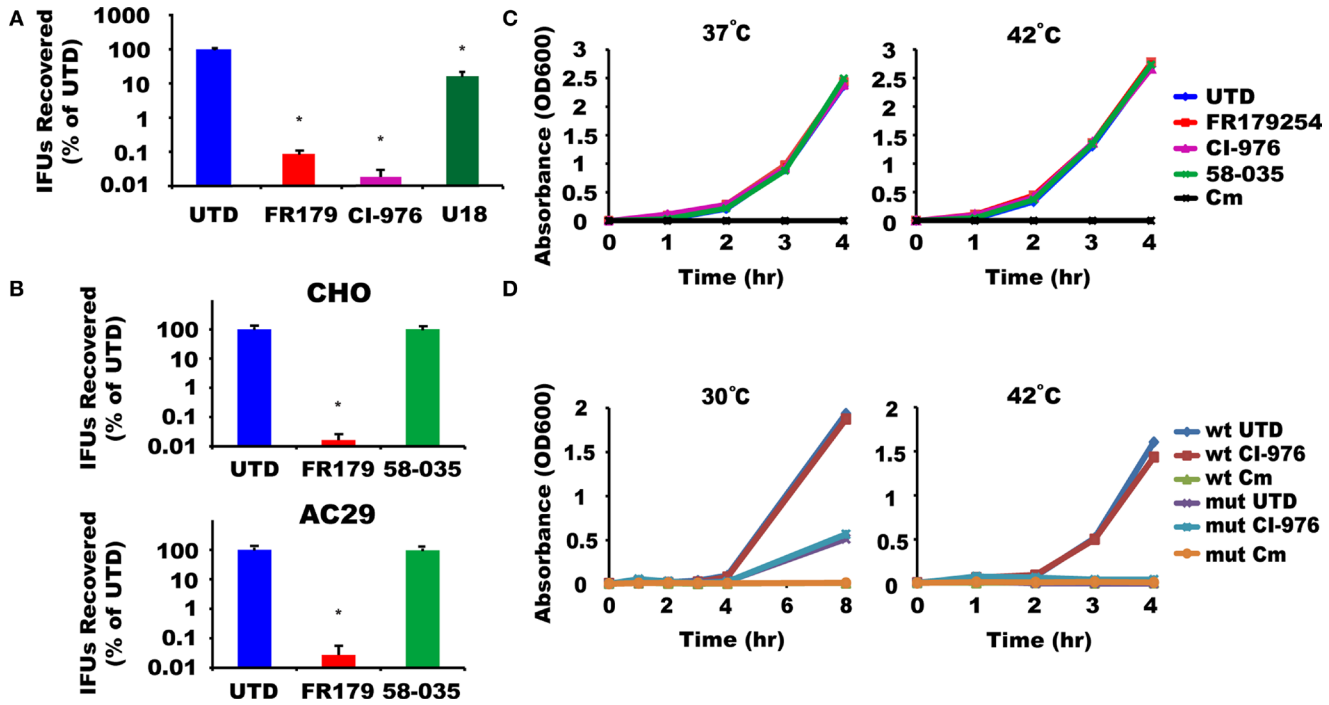


FIGURE 1 | FR179254 blocks chlamydial growth by targeting host cell LPAT and not ACAT or bacterial enzymes. (A) FR179254 (FR179) and CI-976 block chlamydial growth. Effect of indicated treatments on recovery of inclusion forming units (IFUs), a measure for infectious EBs. **(B)** Chlamydiae grow in the absence of ACAT activity. CHO or AC29 cells, a CHO cell mutant which lacks ACAT activity, were infected with *Chlamydia trachomatis* serovar L2 and treated with or without FR179254 or the ACAT inhibitor 58-035 and recovery of infectious EBs was monitored after 24 h infection. **(C)** Assay for LPAT inhibition by compounds in *Escherichia coli*. Wild-type *E. coli* MG1655 were grown in broth for 4 h in the presence or absence of LPAT and ACAT inhibitors or chloramphenicol (Cm) as indicated, and growth was monitored by OD_{600} at 37°C or 42°C. **(D)** Assay for effect of compounds on Δ*plsC* mutant. Wild-type or mutant bacteria were grown in broth for up to 8 h in the presence or absence of the LPAT inhibitor CI-976 or chloramphenicol and growth monitored by OD_{600} at 30°C or 42°C.

FR179254 HAS NO DISCERNIBLE EFFECT ON LIPID DROPLETS, ENDOCYTIC, EXOCYTIC, OR RETROGRADE TRAFFICKING IN TREATED CELLS

The chlamydial inclusion is a pathogen-designed organelle with limited interaction with host vesicular trafficking pathways. However, this image of limited fusogenicity is being challenged by a number of reports that describes inclusion interactions with select Rab GTPases (Cortes et al., 2007; Rzomp et al., 2003, 2006), which are important for vesicular traffic. Displaying a broader range of vesicular interactions, especially transport compartments that are nutrient-laden, would maximize the potential for robust growth. To determine how FR179254 inhibited chlamydial growth, we screened a number of intracellular trafficking pathways using fluorescent microscopic techniques. In particular we sought a phenotype unique to FR179254 by comparing it to untreated and U18666a-treated cells. To initiate these studies, we assessed lipid droplets in untreated and treated cells (**Figure 2A**). FR179254 treatment, in spite of its inhibitory effects on chlamydial growth did not result in a change in lipid droplet contents. Furthermore, inclusions were present in cells with no detectable lipid droplets. Thus, growth inhibition could not be attributed to effects of the compound on lipid droplets. A converse experiment was performed in which lipid droplets were induced in the presence of FR179254, in an effort to rescue chlamydial growth. We observed no such rescue when compared to the untreated group (**Figure 3**).

We continued our studies by examining endocytosis by monitoring fluid-phase uptake as measured by Alexa488-dextran labeling of endocytic compartments. In untreated cells, strong labeling could be seen whereas fluorescence was slightly reduced in both the FR179254- and U18666a-treated cells (**Figure 2B**). The similar effects on dextran labeling in the two samples did not correlate with the different effects of the two compounds on chlamydial growth, and thus, fluid-phase uptake could be excluded as the targeted process.

We next examined CD63-GFP expressing cells, as a marker for late endosome/multivesicular bodies, and LAMP1-transfected cells as a marker for the lysosomal compartment. We saw no obvious differences in the localization of these markers in FR179254-treated or untreated cells, but U18666a induced the accumulation of these markers (**Figures 2C,D**). Contrary to a published study (Beatty, 2006), we did not see significant CD63 localization in the chlamydial inclusion under any condition examined unless we fixed and permeabilized the cells following the methods of Beatty (2006; data not shown) for immunofluorescence confocal microscopy: our CD63-GFP cells were visualized live. We concluded from these experiments that the *Chlamydia*-relevant transport pathway targeted by FR179254 was unlikely to be the endocytic pathway.

We next assayed the exocytic pathway by monitoring the transport of sphingolipid and glycoprotein cargos to the inclusion and plasma membrane, respectively. Many studies have confirmed

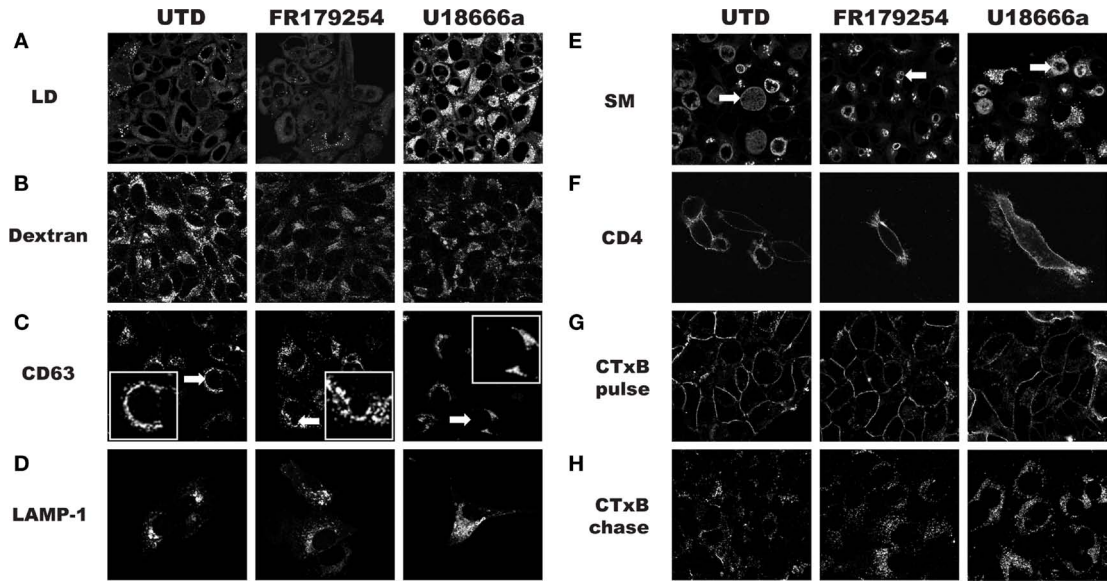


FIGURE 2 | Analysis of host cell trafficking pathways during treatment with FR179254. HEp-2 cells were treated as indicated, and trafficking pathways were analyzed as described in Section "Materials and Methods." **(A)** Lipid droplet (LD) visualization with the neutral lipid stain Bodipy 493. **(B)** Fluid-phase uptake as measured by fluorescent dextran. **(C)** Live cell visualization of multivesicular bodies/late endosomes using CD63-GFP expressing HEp-2 cells. **(D)** Analysis of late endosomes/lysosomes in LAMP1-YFP transfected cells. **(E)** Exocytic trafficking as measured by incorporation of fluorescent sphingomyelin (SM) in Golgi and chlamydiae. **(F)** Exocytic trafficking of glycoproteins as measured by localization of CD4 on the surface of transfected cells. **(G,H)** Analysis of retrograde trafficking using fluorescently tagged cholera toxin subunit B (CTxB) after pulse-chase labeling. Arrows within **(C)** and **(E)** indicate inclusions. Note the lack of significant staining in **(C)** (inset) and the presence of staining in **(E)**, which is contrast to a published study (Beatty, 2006).

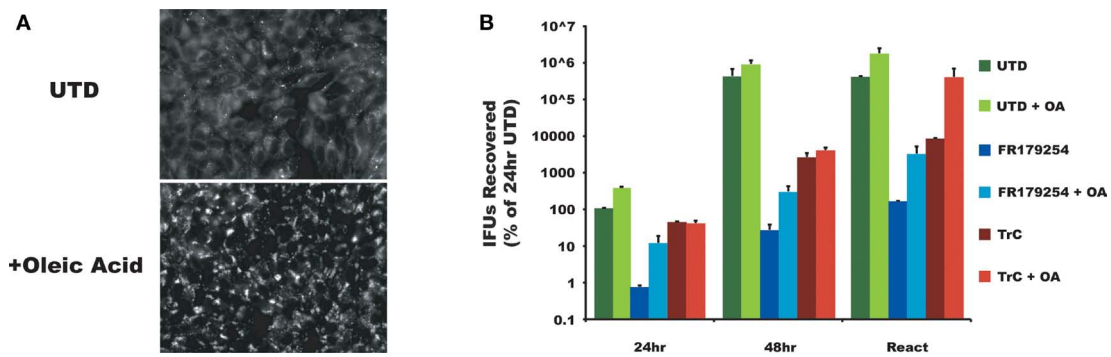


FIGURE 3 | Excess oleic acid fails to reverse the effect of FR179254. Lipid droplets are not the targeted process of FR179254. Cells were cultured in normal medium or medium containing excess oleic acid (OA) to induce lipid droplets **(A)** and then infected and treated as indicated with either FR179254 or Triacsin C (TrC), a fatty acid synthase and lipid droplet inhibitor. **(B)** Chlamydial growth was assessed at 24 and 48 h post-infection. One set of samples were reactivated (React) by treating with indicated drugs for 24 h, washing three times, and then culturing in the presence or absence of excess oleic acid in fresh medium lacking drugs. Reactivated samples were collected after 24 h (48 h total culture time). All data were normalized to chlamydial growth in untreated cells at 24 h post-infection (arbitrarily 100%).

the observation that SM-containing vesicles are trafficked to the inclusion via a novel route that excludes glycoproteins (Scidmore et al., 1996a). To better aid visualization, we delayed treatment with drugs until 8 h post-infection to allow for inclusion development. When we monitored SM trafficking to the inclusion at 24 h post-infection in untreated and treated cells by live cell microscopy, we observed no defect in labeling of the chlamydiae (**Figure 2E**). This is in contrast to a report by Beatty (2006) that suggested U18666a blocked the trafficking of SM to the inclusion during observation of fixed cells. The reason for this discrepancy is not known and warrants further investigation, but may be explained by the difference in methodology vis-à-vis live cell imaging versus observation of fixed cells. To verify that trafficking of proteins to the cell membrane was not affected by treatment, we monitored CD4 or vesicular stomatitis virus G-glycoprotein (VSV-G) transfected, but uninfected cells (**Figure 2F**; data not shown). Transfected cells were pre-treated with drugs where indicated and maintained in the presence of drugs after transfection. CD4 and VSV-G was visualized on the cell surface

without permeabilization of the cells. Here too, we saw no defects in labeling and concluded that FR179254 is not affecting the exocytic pathway in a way that impacts chlamydial growth.

Another prominent intracellular trafficking pathway is the retrograde transport, which allows for certain endocytosed molecules to be diverted to the exocytic pathway where they transit back to the ER. We examined the retrograde trafficking of fluorescently tagged cholera toxin subunit B (CTxB) in untreated and treated cells. After an initial pulse, cells showed good labeling of the membrane, regardless of the conditions used (**Figure 2G**). Cells were then chased in medium for 2 h, fixed, and viewed by confocal microscopy. We did not observe any noticeable differences between untreated and FR179254-treated cells (**Figure 2H**). We concluded that retrograde transport was unlikely to be the sought-after target of FR179254. It appears that FR179254 treatment did not result in any qualitative differences in the staining profile and morphology of the compartments within the endolysosomal, exocytic, and retrograde transport pathways.

FR179254 INDUCES A DELAY IN THE SLOW TRANSFERRIN RECYCLING PATHWAY

Because Tf has been localized to the chlamydial vacuole (Scidmore et al., 2003; van Ooij et al., 1997), we examined Tf recycling in treated and untreated cells. Cells were pulse-labeled with fluorescent Tf for 1 h at 4°C to prevent uptake, washed, and shifted to 37°C to allow uptake and recycling. Labeling of cells was unaffected by treatment. Surprisingly, FR179254 induced a marked retention of Tf compared to untreated or U18666a-treated cells that was both quantifiable and easily seen in fluorescent images (**Figure 4A**). We also found that Sandoz 58-035 and lipid droplet inhibitors had no effect on Tf recycling (data not shown). Thus, it seems that the growth inhibitory effects of FR179254 on chlamydiae are due to its ability to delay Tf recycling.

To further characterize the effects of FR179254 on Tf recycling, we monitored retention of fluorescent Tf within treated and untreated cells over a time course during the chase period. Based on the distinct differences in Tf retention of the untreated and FR179254-treated samples (**Figure 4A**), the use of fluorescent Tf rather than radioactively labeled ones was deemed sufficient and informative. Indeed, we observed that the bulk (approximately 80%) of endocytosed Tf is rapidly recycled back to the plasma membrane within 30 min whereas the remainder is recycled more slowly. Up to 30 min chase, there were no significant differences between retained fluorescence (**Figures 4B,C**), marking the initial uptake and bulk recycling of Tf through the fast recycling pathway. However, after 30 min, we noticed obvious, significant retention of labeled Tf within treated cells, which corresponds with the slow pathway of Tf recycling (**Figures 4B,C**). We concluded from these

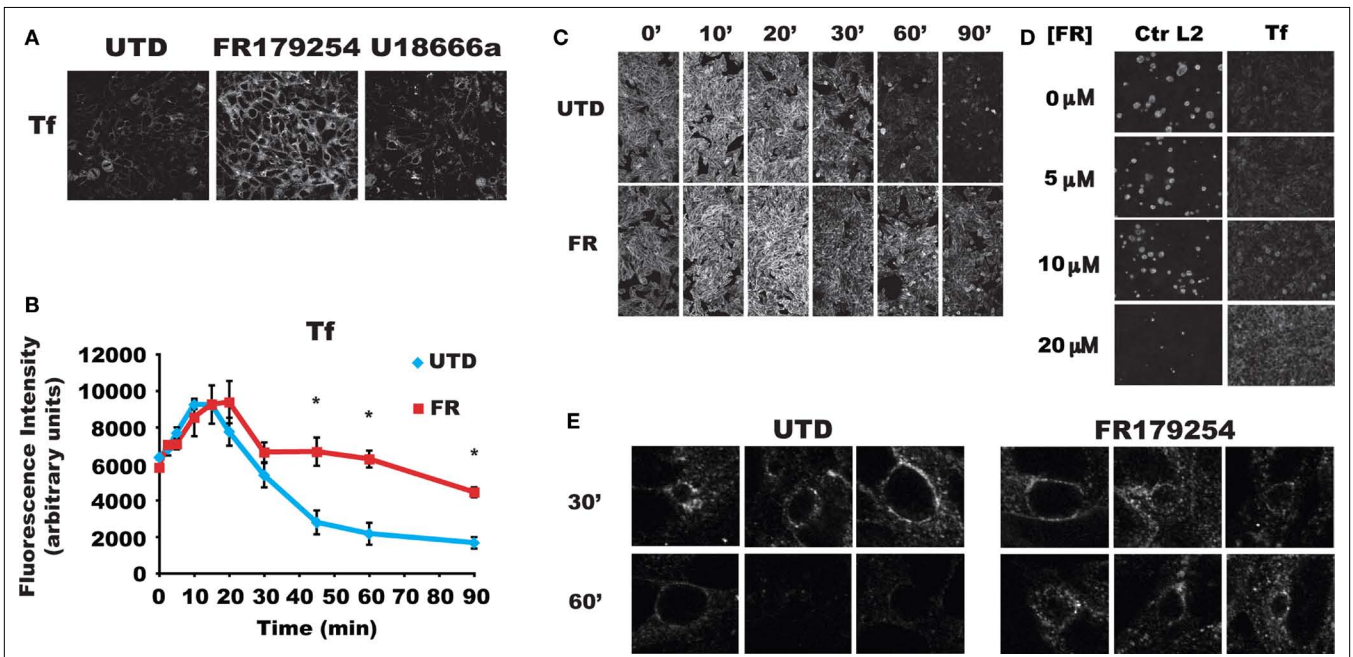


FIGURE 4 | Slow transferrin recycling is delayed in FR179254 treated cells. HEp-2 cells were treated as indicated, pulsed with fluorescent transferrin, and chased for the times shown. **(A)** Fluorescent micrographs of transferrin pulse-chased cells from different conditions at 600× magnification acquired by laser scanning confocal microscopy using identical settings. **(B)** Quantification of transferrin fluorescence in treated cells measured over a time course during chase. **(C)** Representative images used for quantification. FR179254 shows no effect on transferrin recycling during the early (up to 30′) part of the chase but significantly delays recycling during the latter phases. "*" Indicates $p < 0.005$ compared to UTD calculated by Student's *t*-test. **(D)** HEp-2 cells were infected and treated with different concentrations of FR179254 as indicated and processed for immunofluorescence of chlamydial inclusions after 24 h infection. Separate samples were treated as indicated and pulse-chased with fluorescent transferrin as described previously and imaged at 60′ chase time. Chlamydial inclusions were imaged at 400× magnification whereas transferrin staining was imaged at 100× magnification using the same settings on the microscope. Images were acquired on an epifluorescent microscope. **(E)** Transferrin is recruited to the inclusion periphery and remains there for an extended time during FR179254 treatment. Cells were infected and treated with or without FR179254 at 12 h post-infection to allow inclusions to develop for easy visualization. Cells were then pulse-chased with fluorescent transferrin as described at 24 h post-infection.

data that FR179254 specifically affects the transit of Tf through the slow recycling pathway while having no effect on the fast recycling pathway.

To correlate the delay in Tf recycling with chlamydial growth, we monitored chlamydial inclusion development and Tf recycling over a dose curve of FR179254. Chlamydial inclusion formation was significantly reduced at a concentration of 20 μM, accompanied by the marked retention of fluorescent Tf in parallel samples (**Figure 4D**). Lower concentrations failed to show an obvious microscopic effect on either chlamydial inclusions development or Tf retention, although 10 μM FR179254 inhibited chlamydial recovery by approximately 0.5 log (data not shown). An excellent concordance was seen between the effect of FR179254 on Tf recycling and chlamydial inclusion formation, supporting the notion that interference with the Tf recycling pathway was linked to chlamydial growth.

We next examined the effects of FR179254 treatment on the subpopulation of Tf that localize around the chlamydial inclusion. Would they be refractory to the inhibitor, and thus belong to the fast/bulk recycling, or would they be retained similarly as the Tf in the slow recycling pathway? We analyzed confocal images of treated and untreated infected cells. As others have reported (Al-Younes et al., 1999; Rzomp et al., 2006; Scidmore et al., 1996b, 2003; van Ooij et al., 1997), we could detect Tf around inclusions at 30 min chase time (**Figure 4E**). By 60 min, most of this staining was absent, indicating that the Tf had been recycled away from the inclusion, apparently with a kinetics consistent with that of slow recycling. Treatment with FR179254 resulted in the retention of Tf around the chlamydial inclusion at 30 and 60 min chase times (**Figure 4E**). Based on the susceptibility of this peri-inclusion Tf to FR179254 treatment, it is very likely that this Tf subpopulation belong to the slow pathway of recycling.

MULTIPLE INFLUXES OF TRANSFERRIN ARE DELAYED BY FR179254 TREATMENT

If a pulse of labeled Tf was found to be retained for a longer duration, it would be logical to assume that under steady state labeling or even growth in Tf-rich serum would result in accumulation. To investigate further the apparent retention of Tf, we performed a double-pulse-chase experiments using Tf conjugated to different fluorophores. Untreated and treated cells were initially pulsed with Alexa488-Tf, and after 30 min chase time, were pulsed again with Alexa647-Tf and chased for a further 60 min. We found that each fluorescence label was retained in FR179254-treated cells with similar kinetics (see **Figure 4**) whereas, in untreated or Sandoz 58-035-treated cells, the fluorescence associated with both Tf pulses was lost quite rapidly (**Figure 5**). These results are consistent with a chronic delay of Tf recycling through the slow pathway such that each wave of Tf endocytosed by the cell is similarly affected.

FR179254 TREATMENT INHIBITS THE TRANSPORT OF TRANSFERRIN TO THE RAB4/11 HYBRID RECYCLING VESICLE

The previously reported effects of CI-976 include delaying the recycling of Tf, and causing the accumulation of Tf in Rab11-positive compartments (Chambers et al., 2005). Therefore, we sought to determine if the same is true for FR179254. Also, we extended this observation by including in the analysis the Rab4/5 and Rab4/11 hybrid vesicle intermediates that may function in sorting the Tf/Tf receptor from the endosomal compartments for recycling to the plasma membrane via the fast and slow recycling pathways, respectively. Cells were transfected with one of three markers either individually or in combination with Rab4 (a marker for recycling vesicles): Rab5 or 2x-FYVE domain (early endosome markers) or Rab11 (late endosome/endocytic recycling compartment). Cells were infected and then pulse-chased with Tf and processed for confocal microscopy. The number of vesicles co-localizing with Tf were quantified.

In untreated cells, Tf-containing vesicles co-localizing with the singly transfected markers increase between the end of the Tf pulse and 30 min chase (**Figure 6A**) and decline to "starting" levels by 60 min chase (**Figure 6B**). A similar pattern was seen for Tf co-localizing with the FYVE domain in FR179254-treated cells. In contrast, Tf-positive Rab4 vesicles did not decrease after 30 min, and Tf-positive Rab11 vesicles accumulated over time (**Figures 6A,B**), consistent with published effects of LPAT inhibition (Chambers et al., 2005). To further characterize the compartment in which Tf was delayed, we monitored doubly transfected cells for Tf localization. There were no differences in Tf localization in Rab5/Rab4 doubly positive compartments between untreated or FR179254-treated cells (**Figures 6C,D**). Curiously, there was a marked decrease in the levels of Tf in FR179254-treated Rab4/Rab11 doubly positive compartments (**Figures 6C,D**). However, assessing singly transfected Rab11 positive cells within the same cell population revealed that this deficit in Tf/Rab4/Rab11 co-localization was compensated by an increase in the levels of Tf/Rab11 co-localization compared to untreated cells (**Figure 6**). These data indicated that there was a defect in the formation of the Tf-containing Rab4/11 hybrid vesicles. These same observations applied to infected cells, but enumeration of vesicles was difficult due to the vesicles around the inclusions being less distinct or sometimes appearing fused (data not shown). Our observation of Tf transiting through the Rab4/11 hybrid vesicle on its way to the plasma membrane concurs with the model of Tf recycling proposed (Sönnichsen et al., 2000).

CO-EXPRESSION OF DOMINANT-NEGATIVE CONSTRUCTS FOR RAB4 AND RAB11 CAUSES RETENTION OF TRANSFERRIN AND DELAYS INCLUSION DEVELOPMENT

Previous reports investigating the ability of dominant-negative Rabs to inhibit chlamydial growth failed to show any biologically significant effects (Rzomp et al., 2006; Rejman Lipinski et al., 2009). Possible explanations for this include the compensatory functions provided by the wild-type, endogenous proteins and/or the ability of chlamydial proteins to selectively interact with them (Rzomp et al., 2006). Given the apparent involvement of both Rab4 and Rab11 in the slow recycling pathway, we speculated that co-expression of both dominant-negative Rab4 and Rab11 may lead to Tf retention and chlamydial growth inhibition. Cells were transfected with both constructs, infected, and fixed after 24 h. Tf retention and inclusion sizes were respectively monitored and measured in untransfected, singly transfected, and doubly transfected cells within the same population. For the Tf experiment, chlamydiae-infected cells were incubated with Alexa647-Tf for a 1-h pulse, and chased for 90 min. **Figure 7A** displays a representative visual field with cells harboring chlamydial inclusions (white arrowhead). Only cells that expressed both the dominant-negative forms of Rab4

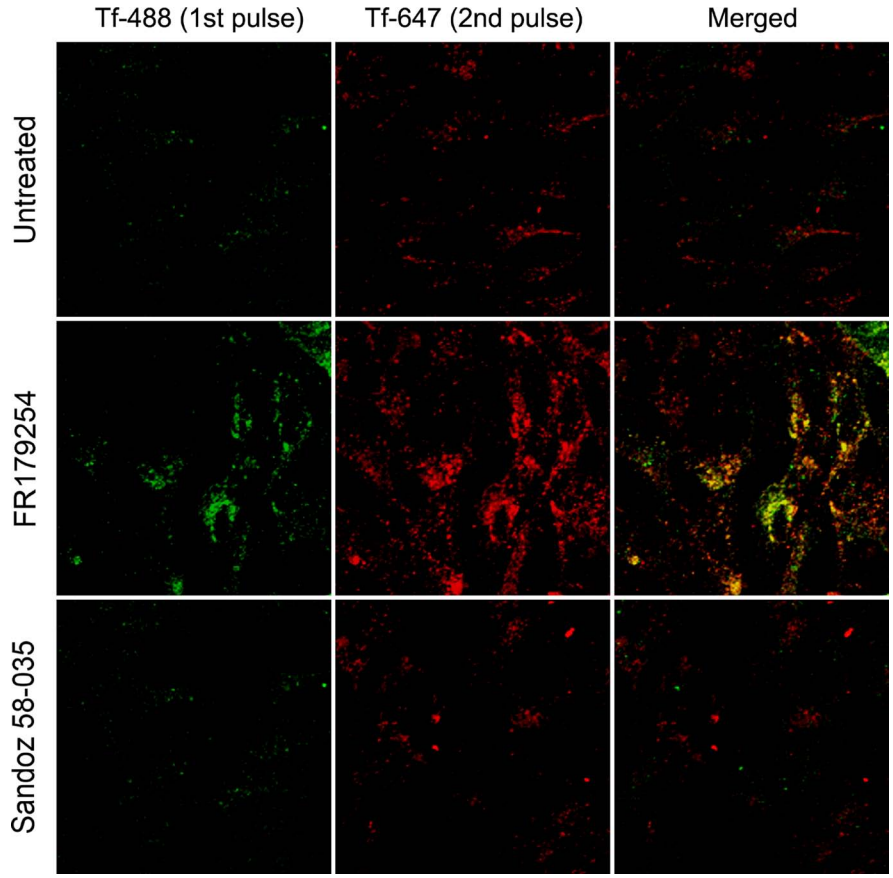


FIGURE 5 | Successive waves of internalized transferrin are delayed by FR179254 treatment. Cells were treated as indicated and pulse-chased with Tf-488. At 30′ chase, cells were subsequently pulsed with Tf-647. After a total of 90′ chase time (60′ for Tf-647), cells were fixed and analyzed by confocal microscopy. Noticeable accumulation of both fluorescent labels is present in FR179254-treated but not Sandoz 58-035-treated or untreated cells.

and Rab11 specifically retained Tf that circumscribed the inclusions ($p < 0.002$; chi-square test) whereas expression of Rab4 or Rab11 DN only led to a mild retention of Tf fluorescence and untransfected cells showed very little Tf retained around the inclusion. Thus, much like FR179254, the co-expression of the dominant-negative mutants of Rab4 and Rab11 led to relatively higher levels of retained Tf when compared to untransfected cells.

As shown previously, singly transfected cells expressing either Rab4 or Rab11 dominant-negative GTPases failed to significantly reduce chlamydial inclusion size, a correlate for IFU output. Similarly, the dominant-negative constructs failed to circumferentially stain the inclusion. However, inclusion sizes in cells expressing both dominant-negative GTPases were significantly reduced albeit not to the same extent as for FR179254-treated cells (**Figure 7B**). In contrast to co-expression, the lone expression of each dominant-negative mutant did not result in a statistically significant decrease in inclusion size, suggestive of a synergistic effect of co-expression (**Figure 7C**).

FR179254 INHIBITION OF GROWTH OF *CHLAMYDIA* IS REVERSED BY OMITTING SERUM FROM THE MEDIUM

We have shown above that FR19254 treatment resulted in the significant retention of Tf (**Figure 5**), and that the Tf retention correlated with decreased inclusion development (**Figure 4D**). This correlation was also observed in dominant-negative transfection experiments (**Figure 7**). If Tf accumulation was the primary cause of growth inhibition of *Chlamydia* by FR179254, we reasoned that omission of Tf from the growth media would relieve this inhibition. We monitored chlamydial growth in media lacking whole serum or supplemented only with the Tf-containing fraction of the serum in FR179254-treated and untreated cells. An SDS-PAGE and Western blot analysis of the serum fractions (less than 50 kDa, 50–100 kDa, and greater than 100 kDa) compared to purified Tf or complete serum demonstrated that the fraction with the greatest amount of Tf was the >100 kDa fraction (**Figure 8**), owing to the tendency of native Tf proteins to aggregate during the concentration process. In the absence of serum, FR179254 had no effect on chlamydial growth, as measured by IFU recovery and visualized by fluorescence microscopy of inclusions (**Figure 8** and data not shown). Consequently, the supplementation of the Tf-containing fraction of the serum to the growth media restored the inhibitory effects of the compound (red bars in **Figure 8** indicate no significant difference compared to FR). No such restoration of inhibition could be observed when other fractions of the serum were used individually (purple bars represent a significant difference in growth compared to FR treatment; **Figure 7**). Therefore, we concluded

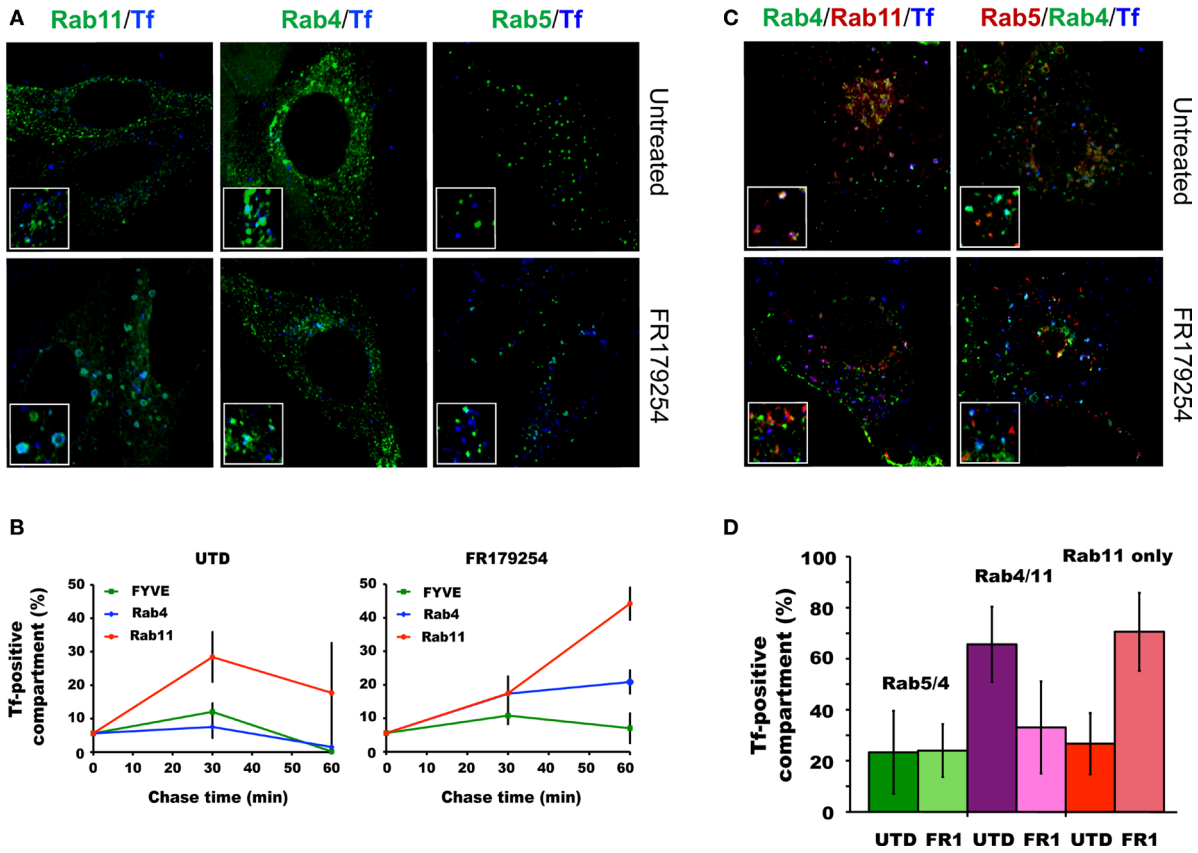


FIGURE 6 | FR179254 causes a delay in the trafficking of transferrin from Rab11 compartments to Rab4/11 recycling compartments. Transfected cells were pulse-chased with fluorescent transferrin (Tf647) as described previously and monitored for co-localization with vesicles by confocal microscopy. Representative confocal images **(A)** and quantification **(B)** of co-localization of transferrin with individual markers in singly transfected cells in untreated or FR179254-treated conditions. Representative confocal images **(C)** and quantification **(D)** of co-localization of transferrin with indicated markers in doubly transfected cells.

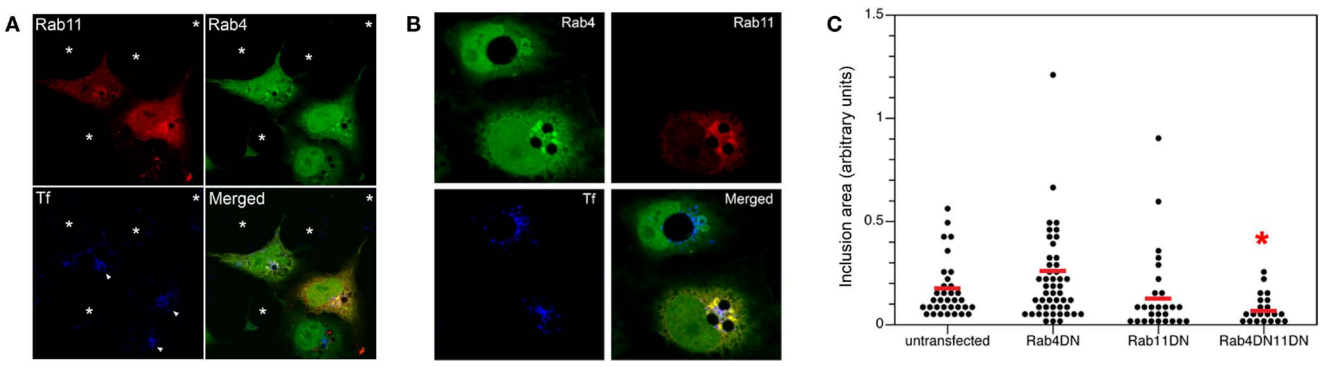
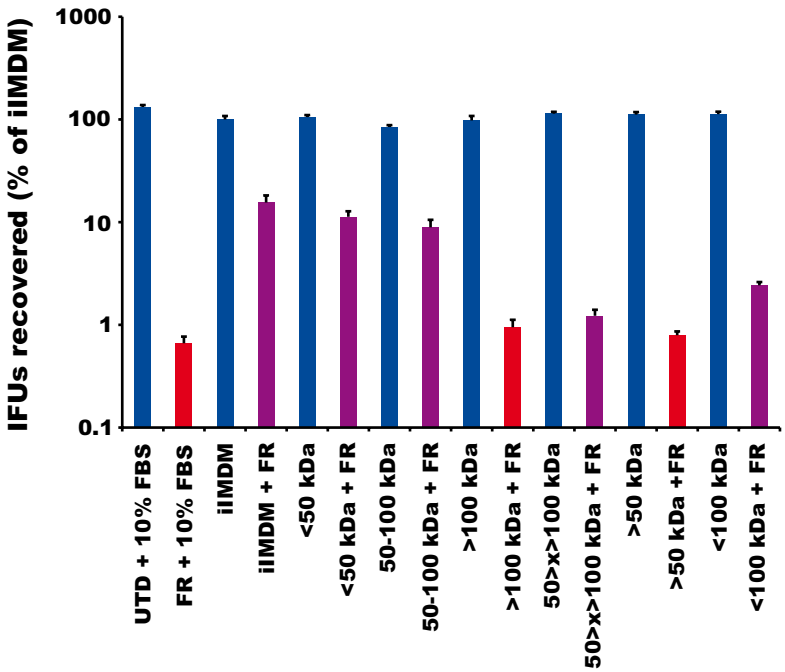


FIGURE 7 | Co-expression of dominant-negative constructs of Rab4 and Rab11 GTPases causes retention of Tf and reduces chlamydial inclusion growth. Cells were transfected with dominant-negative (DN) constructs of Rab4 or Rab11 either individually or in conjunction. **(A)** Transfected cells were pulse-chased with fluorescent Tf to monitor Tf retention in these cell types. **(B,C)** Quantification of inclusion size in cells transfected with indicated construct(s) with accompanying representative confocal image. * indicates $p < 0.001$ compared to UTD by Kruskal–Wallis test.

that the inhibitor activity of FR179254 only manifested in the presence of Tf in the growth media. An equally important result obtained from this experiment is the normal growth of chlamydia in media lacking serum, but containing the inhibitor, which supports our data above (**Figures 1 and 4**) that the target of the compound is not the bacteria.



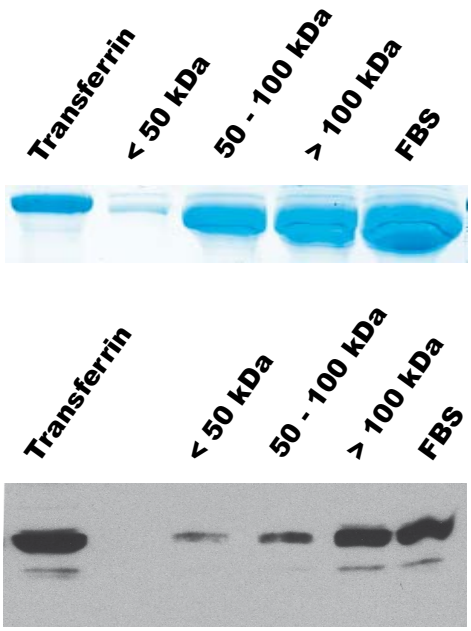


FIGURE 8 | The effect of FR179254 can be reversed by omission of the transferrin-containing fraction of serum from the medium. Infected cells were treated with or without FR179254 (FR) and cultured in the presence or absence of serum or serum fractions as described in Section "Materials and Methods." IFUs were collected and quantified. Red bars indicate no statistically significant difference compared to FR treatment, hence restoration of inhibition; whereas purple bars indicate a significant difference ($p < 0.05$ by Student's *t*-test). SDS-PAGE analysis and Western blot of serum fractions for Tf content compared to complete serum (FBS) and purified, commercially available Tf.

DISCUSSION

Chlamydia is a medically important pathogen that has proven difficult to genetically manipulate, thus alternative methods must be employed to investigate its biology at a mechanistic level. We used a small molecule inhibitor approach to identify chlamydial and host processes required for survival of the pathogen, and here, we report the identification of an inhibitor of LPAT, which interrupted the proper recycling of Tf – specifically via the slow recycling pathway. In the context of *Chlamydia*-infected cells, interference of this pathway using the small molecule inhibitor suppressed chlamydial growth, and thus providing additional insight into the significance of the oft-reported localization of Tf around the inclusion.

The chlamydial inclusion lacks markers of the endolysosomal pathway and is distinct from the phagosomes of other intracellular bacteria, which, aside from *Coxiella*, appear to be arrested within the endolysosomal transport pathway (Scott et al., 2003). This unusual characteristic of the inclusion was reinforced by the reported interaction with exocytic Golgi-derived vesicles (Hackstadt et al., 1996; Carabeo et al., 2003), and the localization to the inclusion of Rab GTPases associated with various intracellular trafficking pathways challenged this notion of restricted fusogenicity (Rzomp et al., 2003). In this report we demonstrated the requirement for the proper recycling of Tf through the slow recycling pathway in inclusion development and the completion of the developmental cycle of *Chlamydia*. A model is proposed below demonstrating the potential interaction of the chlamydial inclusion with the recycling pathway (**Figure 9**). The preferential interaction with the slow recycling pathway is supported by our FR179254 experiments.

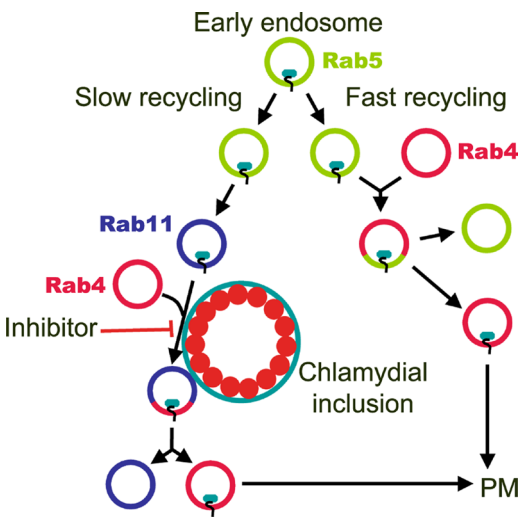


FIGURE 9 | A model for the action of FR179254 against chlamydiae. Transferrin is recycled through two main pathways. For fast recycling, the majority of transferrin (~80%) is endocytosed into a Rab5 positive early endosome, which subsequently fuses with Rab4 recycling endosomes for transport back to the plasma membrane (PM) in Rab4 vesicles. For slow recycling, transferrin (~20%) is endocytosed into Rab5 early endosomes, which mature to Rab11 compartments that then fuse with Rab4 for recycling back to the PM. FR179254 delays the fusion of transferrin-containing Rab11 vesicles with Rab4 recycling vesicles, possibly at the inclusion membrane, leading to chlamydial growth abrogation.

The negative effects of LPAT inhibition on the slow recycling pathway has been reported previously (Chambers et al., 2005), and here, we extend it to *Chlamydia*-infected cells. We observed that the previously reported accumulation of Tf in Rab11 vesicles was at the expense of Tf localizing to the Rab4/11 hybrid transport intermediate, which Sönnichsen et al. (2000) have proposed to be involved in the recycling of Tf to the plasma membrane. This defect in the slow recycling pathway could also be observed in infected cells, with Tf accumulating around the inclusion. We suspect that the Tf accumulation is directly linked to inhibition of chlamydial growth because the removal of the Tf-containing fraction from the serum alleviated the growth inhibitory effects of FR179254. It is possible that chlamydia may have requirements for Tf-independent recycling pathway. However, the near-complete restoration of the inhibition of chlamydial growth of FR179254 treatment by the simple addition of Tf-containing fractions indicate that Tf accumulation contributes significantly to inhibition of chlamydial growth. This observation is also important in demonstrating that the target of the anti-chlamydial activity of this compound is of host origin; otherwise, alleviation of growth inhibition would not have been observed in the absence of Tf, while in the continued presence of the inhibitor. We could not observe a similar growth inhibition using commercially available purified holo-Tf (unpublished observation) either due to the difficulty in reaching the Tf concentration level found in the serum or the possibly insufficient level of iron loading of the commercial preparations of holo-Tf. Further evidence for a host target is our demonstration that the co-expression of the dominant-negative mutants of Rab4 and Rab11 partially mimicked LPAT growth inhibition of chlamydial inclusions, and that the prokaryotic LPAT homolog, 1-acylglycerol-3-phosphate acyltransferase encoded by *plsC* in *E. coli* was refractory to the inhibition of FR179254. Prokaryotes have an enzyme with predicted LPAT-like activity (Zhang and Rock, 2008). This enzyme participates in the synthesis of phospholipids from phosphatidic acid. Given the conservation of this pathway among prokaryotes and the relatively high levels of homology of the enzymes within this biosynthetic pathway, it is likely the inhibitor would be similarly ineffective toward *Chlamydia*. Furthermore, the PlsC pathway may be dispensable in chlamydia, as work by Su et al. (2004) demonstrated that chlamydia uses host cell precursors to generate phospholipids, thus circumventing any requirement for the Pls system.

How the inhibition of the host cell LPAT links to defects in the slow recycling pathway is unknown. There are at least eight LPAT isoforms that display differential subcellular localization (Shindou and Shimizu, 2009), and Brown and colleagues have reported (Chambers and Brown, 2004) that the relevant LPAT targeted by CI-976, which we also showed to have similar effects with regards to chlamydial infection and Tf transport as FR179254, is the Golgi-associated isoform. Because the Golgi apparatus is involved in the biogenesis of vesicles in the endosomal compartments, it is possible that interference with the Golgi-associated LPAT may affect the Tf-containing Rab11 compartment. However, we did not observed defects in the transport to the plasma membrane of protein (CD4 and VSV-G) cargos, or the delivery of SM to the chlamydial inclusion. It is tempting to speculate that a drastic change induced by LPAT inhibition in the general lipid composition of intracellular compartments may contribute to the delayed recycling of Tf via the slow pathway. However, this possibility seems unlikely as transport of other cargos via the endocytic, retrograde, and anterograde pathways seemed unaffected in cells treated with the LPAT inhibitor. The apparent specific inhibition of Tf recycling by the LPAT inhibitors may be due to unique properties of the slow recycling compartment, possibly vesicle tubulation, which is critical for recycling, or the localization of critical proteins to the slow recycling vesicles. The molecular link between LPAT and Tf transport requires further investigation.

How might the slow Tf recycling pathway contribute to chlamydial growth? The lack of obvious siderophore homologs necessitates the capture of iron prior to release to the host cytosol, where the metal is immediately sequestered by host ferritin. Such a model would imply the presence of iron in the slow recycling pathway. Indeed, the divalent metal transporter 1 (Dmt1) has been localized to the endocytic recycling compartments (Tabuchi et al., 2000), suggesting that iron may be present in these vesicles. The close association of the inclusion with these Rab11-positive vesicles would allow *Chlamydia* access to a possible iron source, perhaps by transient "kiss-and-run" interaction as has been suggested for phospholipid acquisition (Hatch and McClarty, 1998). In the presence of LPAT inhibitors, the resulting retention and subsequent accumulation of iron-laden compartments around the chlamydial inclusion would lead to iron overload and toxicity. Preliminary results indicate that chlamydial genes induced under limiting iron availability were, in contrast, repressed under conditions of iron overload. Thus, we are now investigating the possibility of the slow recycling pathway as the source of iron *Chlamydia* and that the dysregulation of its recycling, such as that induced by the LPAT inhibitors results in the uncontrolled transfer of potently oxidative Fe^{2+}. Because of the deleterious effects of a severely delayed transport of Tf through the slow recycling pathway, any interaction the inclusion may have with this trafficking compartment would need to be regulated by *Chlamydia* to ensure minimal interference with the recycling of Tf to the plasmamembrane.

As with most living organisms, chlamydia absolutely requires iron. Iron limitation under *in vitro* conditions induces aberrant growth, growth arrest, and a transcriptional response in chlamydia (Timms et al., 2009; Wehrl et al., 2004; Wyrick, 2010). *In vivo*, interferon-γ, being a potent anti-chlamydial cytokine also modulates iron acquisition of and metabolism in the host to starve intracellular pathogens of this essential metal (Beekhuizen and van de Gevel, 2007; Sow et al., 2007; Nairz et al., 2008). Hence, iron limitation induced by this cytokine could act in concert with other anti-bacterial effectors to effectively eliminate infection. It is tempting to speculate that the slow recycling compartment could be the source of iron that chlamydia utilizes. By interacting directly with this pathway, chlamydia bypasses the need to compete with host cytosolic ferritins. This mechanism of iron acquisition would be especially important for a non-siderophore synthesizing intracellular pathogen.

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An optimal method of iron starvation of the obligate intracellular pathogen, *Chlamydia trachomatis*

Christopher C. Thompson and Rey A. Carabeo*

Division of Cell and Molecular Biology, Centre for Molecular Microbiology and Infection, Imperial College London, London, UK

Edited by:
Kenneth Fields, University of Miami, USA

Reviewed by:
Georg Hacker, Technische Universität München, Germany
Dan Rockey, Oregon State University, USA

****Correspondence:***
Rey A. Carabeo, Division of Cell and Molecular Biology, Centre for Molecular Microbiology and Infection, Imperial College London, Exhibition Road, London SW7 2AZ, UK.
e-mail: r.carabeo@imperial.ac.uk

Iron is an essential cofactor in a number of critical biochemical reactions, and as such, its acquisition, storage, and metabolism is highly regulated in most organisms. The obligate intracellular bacterium, *Chlamydia trachomatis* experiences a developmental arrest when iron within the host is depleted. The nature of the iron starvation response in *Chlamydia* is relatively uncharacterized because of the likely inefficient method of iron depletion, which currently relies on the compound deferoxamine mesylate (DFO). Inefficient induction of the iron starvation response precludes the identification of iron-regulated genes. This report evaluated DFO with another iron chelator, 2,2′-bipyridyl (Bpdl) and presented a systematic comparison of the two across a range of criteria. We demonstrate that the membrane permeable Bpdl was superior to DFO in the inhibition of chlamydia development, the induction of aberrant morphology, and the induction of an iron starvation transcriptional response in both host and bacteria. Furthermore, iron starvation using Bpdl identified the periplasmic iron-binding protein-encoding *ytgA* gene as iron-responsive. Overall, the data present a compelling argument for the use of Bpdl, rather than DFO, in future iron starvation studies of chlamydia and other intracellular bacteria.

Keywords: *Chlamydia*, iron, deferoxamine, bipyridyl, transcription, persistence

INTRODUCTION

As an obligate, intracellular pathogen, *Chlamydia* has an absolute requirement of the host for essential nutrients. The chlamydial requirement for the biometal, iron, has been well established (Raulston, 1997; Al-Younes et al., 2001). Iron is an essential nutrient for nearly all organisms, as it is utilized as an electron intermediate, cofactor, or prosthetic group in a number of conserved biochemical cellular processes. Its biological importance can be attributed to its role as a transition metal with the ability to accept and donate single electrons as it alternates between the ferric (Fe^{3+}) and ferrous (Fe^{2+}) oxidative states (Hentze et al., 2004). When deprived of iron via long-term treatment using the hydrophilic chelator, deferoxamine mesylate (DFO), *Chlamydiae* enter an alternative growth mode, termed persistence, which was defined as a "viable but non-cultivatable state" of growth (Beatty et al., 1994). Persistence induced by iron starvation is phenotypically similar to those induced by other well-characterized mediators, which include treatment of host cells with IFN-g (Beatty et al., 1993), amino acid limitation (Coles et al., 1993), and B-lactam antibiotic exposure (Matsumoto and Manire, 1970; Lambden et al., 2006), with all exhibiting the accumulation of aberrantly enlarged RBs within the inclusion and the loss of infectious progeny (Beatty et al., 1994; Hogan et al., 2004; Mpiga and Ravaoarinoro, 2006; Wyrick, 2010). Noteworthy is the reversibility of this aberrant phenotype removal of the stress condition (Beatty et al., 1994).

Intracellular pathogens, like *Chlamydia*, are naturally buffered by the host cell from severe fluctuation of iron levels, given the ability of the host cell to maintain intracellular iron homeostasis. This raises the question of whether chlamydia requires, or even possesses iron-dependent regulatory mechanisms of gene expression. Analysis of the chlamydial genome reveals evidence to support the existence of an iron-dependent gene expression – the best studied being *dcrA*, which encodes for a protein that has homology to the prototypical ferric uptake regulator (Fur) protein that regulate transcription of target genes. The exact function of this protein is not known, but it has been demonstrated by Wyllie and Raulston (2001) that it harbors a DNA-binding activity, and that it can bind the same palindromic sequence recognized by *E. coli* Fur. However, further attempts to identify the cis-acting sequence recognized by DcrA in *Chlamydia* was unsuccessful (Rau et al., 2005). Moreover, the lack of a genetically tractable system in *Chlamydia* has precluded the knock-out-mediated identification of the "DcrA-regulon." This lack of further insight is not isolated to DcrA function in *Chlamydia*, but to the entire iron-dependent regulation of gene expression in general. We hypothesize that this paucity in our knowledge of this process could be attributed to a lack of an efficient iron starvation mechanism that overcomes the buffering ability of the cell in relation to the chlamydial organisms it harbors.

The only iron-chelating agent tested against members of the *Chlamydiacea* has been DFO, which binds Fe^{3+} well, but Fe^{2+} poorly (Martell and Smith, 1977). In addition, DFO is membrane impermeable, and thus its chelating ability is restricted to the extracellular media, and perhaps the lumen of the endosomal vesicles (Lloyd et al., 1991; Cable and Lloyd, 1999; Persson et al., 2003; Glickstein et al., 2005). Of the published iron-restriction protocols for the *Chlamydiacea*, the overwhelming majority have utilized DFO with some auxiliary mechanism for iron depletion, including combination with cyclohexamide to block host up-regulation of the transferrin pathway (Raulston, 1997; Dill et al., 2009; Wehrl et al., 2004), or long-term pre-treatment of the host cells prior to infection (Al-Younes et al., 2001; Peters et al., 2005; Mukhopadhyay et al., 2006; Dill et al., 2009; Timms et al., 2009). Taken together,

these published protocols imply that DFO, by itself, may be an unsuitable or inefficient method for the iron starvation of the intracellular pathogen, *Chlamydia trachomatis*. Moreover, the need for additional manipulations raises caveats in data interpretations. Therefore, we sought to identify an alternative means of starving *Chlamydia* of iron with minimal manipulation of the experimental system. We discovered that a single treatment at 100 μM dose at the time of infection with the compound, 2,2′-bipyridyl (Bpdl), which was previously identified as a membrane permeable iron-chelating agent capable of depleting the cytosolic labile iron pool (Breuer et al., 1995; Romeo et al., 2001), proved effective in inducing an iron starvation response in both the host cell and chlamydia. We present evidence that the compound Bpdl is more potent than DFO in inducing an iron-starved, persistent phenotype in *C. trachomatis*. Direct comparison with DFO also revealed a more robust and consistent induction of the transcription of previously reported iron-responsive genes. As final evidence of the superiority of Bpdl over DFO, *ytgA*, which encodes for an iron-binding periplasmic protein was found to be induced in iron-limiting conditions. Thus, we present an iron starvation protocol that requires minimal manipulation, and possesses a potential broader applicability to *Chlamydia* and other intracellular bacteria.

METHODS

CELL CULTURE AND CHLAMYDIAL INFECTIONS

Human endothelial HEp2 cells (CCL-23, ATCC) were grown at 37°C with 5% CO_2 in Iscove's Modified Dulbecco's Medium (IMDM, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS), 20 mM L-glutamine (Gibco), and 10 μg/ml gentamicin. *C. trachomatis* UW-3/CX (serovar D) was originally obtained from Dr. Ted Hackstadt (Rocky Mountain Laboratory, NIAID), and propagated as described (Caldwell et al., 1981).

For infections, HEp2 or HeLa cells were plated to 85–90% confluency in six-well tissue culture treated plates and incubated overnight. Next day, monolayers were treated with 45 μg/ml DEAE-Dextran (Sigma) in HBSS for 10–15 min at 37°C prior to inoculation with *C. trachomatis*. Plates were centrifuged at 1500 rpm at 4°C prior to a 30-min invasion step at 37°C. Inoculum was decanted, cells were washed with PBS, and cultured in complete IMDM (plus or minus iron-chelating agents).

IRON-CHELATING AGENTS

Deferoxamine mesylate (Sigma, CAS: 138-14-7) was diluted to a stock concentration of 50 mM in ddH_2O, filter sterilized and stored at 4°C for no more than 2 months. Bpdl (Sigma, CAS: 366-18-7) was diluted in ethanol to a stock concentration of 100 mM, aliquoted, and stored at −80°C.

QUANTIFICATION OF RECOVERABLE INFECTIOUS PROGENY

HEp2 cells were infected and treated as described. At 24 h post-infection (p.i.; unless noted), infected monolayers were washed prior to physical dislodgement into 1 ml SPG buffer. Suspension was transferred to sterile eppendorf tubes containing three glass beads (3 mm, VWR) and vortexed for 60 s. Samples were stored at −80°C. For quantification, samples were diluted serially and appropriate doses were used to re-infect fresh, confluent monolayers of HEp2 cells in triplicate. The next day, samples were fixed and permeabilized with MeOH and stained with convalescent human sera. Inclusions were visualized after secondary staining with Alexa-594 conjugated mouse mAb anti-human IgG, and counted in five fields per well for the calculation of infectious titer.

IMMUNOFLUORESCENT ANALYSIS AND MICROSCOPY

Infected samples were fixed and permeabilized with MeOH, and immunolabeled with convalescent human sera. Alexa-594 conjugated mouse mAb anti-human IgG was used to visualize chlamydial morphology. Images were captured using Leica SP2 upright confocal microscope. Bars represent 10 μm in each image.

NUCLEIC ACID PREPARATION

Infected monolayers were trypsinized, pelleted, and resuspended in 200 μl PBS for genomic DNA extraction, using the DNeasy Blood and Tissue Kit (QIAGEN) following the instructions of the manufacturer. Samples were eluted in a volume of 100 μl TE buffer.

RNA was extracted using Trizol Reagent (Invitrogen) using the instructions of the manufacturer for cells grown in a monolayer. Extracted RNA was DNAsed using Turbo DNase Kit (Applied Biosystems), precipitated in 80% ethanol overnight, dried, and resuspended in 20 μl nuclease free water (Applied Biosystems). RNA concentrations were measured using Nanodrop ND-1000 spectrophotometer. Equal amounts of total RNA (2 μg) were converted to cDNA using Superscript III reverse transcriptase kit (Invitrogen) according to the instructions of the manufacturer. Reverse transcription was primed using random nonamers (New England Biolabs). cDNA mixture was diluted 1:8 with 10 mM Tris (pH 7.5), for a final volume of 160 μl and stored at −80°C.

REAL TIME QUANTITATIVE PCR (RT-qPCR)

Applied Biosystems 7300 Real time PCR System was used to carry out quantification of cDNA or gDNA sequences. Primers were designed using the Applied Biosystems Primer Express 3.0 Software and are listed in **Table 1**. Primer sets were tested for amplification

Table 1 | Primers used for quantitative PCR.

Gene	Organism	Fwd primer (5′–3′)	Rev primer (5′–3′)
gapdh	*H. sapiens*	ctgctcctcctgttcgacagt	accttccccatggtgtctga
tfr	*H. sapiens*	cattctttggacatgctcatctg	tgatgaccgagatggtggaa
dmt1	*H. sapiens*	ggagtactcttgttttagctttc gtaaa	ccagactgcaaatcggattca
fer-H	*H. sapiens*	tgaagctgcagaaccaacga	cgctctcccagtcatcacagt
fer-L	*H. sapiens*	accgtttttgtggttagctcctt	caggtcggtggaataattctga
euo	*Ctr* serovar D	gctgttcctgttacttcgcaaa	aacatagatagcctgacgag tcaca
omcB	*Ctr* serovar D	ccaaagcgaaagacaacacttct	aaccggagcaacctctttacg
ahpC	*Ctr* serovar D	ccagttagctggacaaacca ttccg	cgttccattgacgaggaat tgcgt
devB	*Ctr* serovar D	acgaagatgtagaagctgg aagta	tgcggtatccatacgaaag atttg
ytgA	*Ctr* serovar D	ctcttgtgtttagcaggctgtttc	tgcgattcatagacaagacat agatg
tyrP-1	*Ctr* serovar D	tcgcaggaacaaccattgg	gtaacgtcgtaggcagga atcc

efficiency against gDNA extracted from purified EBs, and for the generation of specific products using 7300 system post-run Tm function. Samples were assayed in triplicate wells of a 96 well plate; each reaction contained 5 µl DNA (complimentary or genomic), 25 µl 2X SYBR green master mix (Applied Biosystems), 0.45 pmol forward and reverse primers, and 19.8 µl RNase/DNase free water (Applied Biosystems). No RT and no template controls were performed regularly.

Relative expression of eukaryotic iron-responsive transcripts was quantified using the Pfaffl method ($2^{\Delta\Delta Ct}$ method; Pfaffl, 2001). Target iron-responsive transcripts were normalized to *gapdh* transcript levels and calibrated to the untreated sample. Absolute expression of *Chlamydia* transcripts was quantified using serially diluted *C. trachomatis* genomic DNA, which was extracted from EBs, as a standard. Levels of mRNA were normalized to the number of *Chlamydia*-specific genomes, which were derived from parallel samples.

GRAPHS AND STATISTICAL ANALYSIS

Generation of all graphs and statistical tests were performed in GraphPad Prism v5.0 Software. Specific statistical tests are described in the Section "Results" and figure legends. Unless noted, graphs represent mean averages ± SD.

RESULTS

EUKARYOTIC IRON-RESPONSIVE TRANSCRIPTION AFTER IRON-CHELATOR TREATMENT

Intracellular chlamydia are naturally protected from severe fluctuations of extracellular environmental conditions, and thus eliciting a response for the bacterium would first require that the host cell be affected. Two different iron chelators, DFO and Bpdl (shown in **Figure 1**) were evaluated for their ability to induce an iron response from the host cell, with the underlying assumption that iron starvation of the host would lead to iron starvation of intracellular chlamydiae. To assess the level of host iron starvation, we chose to examine the differential regulation of a handful of iron-related transcripts that are known to respond to changes in iron levels. We utilized the mRNA levels of specific iron-responsive transcripts from uninfected HEp2 cells that had been treated with DFO or Bpdl for 10 h (**Figure 2**). The mRNA of two genes coding for iron-acquisitional proteins, transferrin receptor (TfR) and divalent metal transport protein 1 (DMT1) are normally stabilized under conditions of low cytoplasmic iron availability (Rao et al., 1986; Kato et al., 2007), resulting in the net increase in the levels of these transcripts. Conversely, ferritin heavy/light chain transcripts, which code for proteins that form a complex for the storage of excess iron, are normally degraded under the same low-iron conditions (Kato et al., 2007). Based on previous reports of intracellular iron-chelation, we expected Bpdl to cause an iron-starved transcriptional state of eukaryotic cells. Indeed, Bpdl treatment caused an elevated expression of *tfr* and *dmt-1*, which reached 5.2- and 3.5-fold over the untreated sample, respectively (unpaired, one-tailed *t*-test, $p = 0.0054$ and 0.0065, respectively). Moreover, treatment with Bpdl elicited an approximate twofold reduction in ferritin heavy/light chain (*fer-H/L*) transcripts ($p = 0.0255$ and 0.0458, respectively). The ability for Bpdl to cause an "iron-starved" transcriptional response from uninfected HEp2 cells, in a time frame where DFO could not, raised the possibility that Bpdl could induce a relatively robust iron starvation response and/or growth inhibition in *C. trachomatis*.

THE EFFECT OF BIPYRIDYL ON ACUTE *C. TRACHOMATIS* INFECTION

The observation that Bpdl was able to induce a greater iron starvation response from the host cell highly suggested that a similar response could be elicited from intracellular chlamydiae. Therefore we tested both Bpdl and DFO for effects on chlamydial development and inclusion morphology, with an emphasis on the simplest possible iron starvation protocol, which involves a single treatment at the time of infection. In order to test the effect of Bpdl on *C. trachomatis* development, a dose-response analysis of recoverable inclusion forming units (IFU) was performed (**Figure 3**). HEp2 cells were infected with *C. trachomatis* serovar D at an MOI of 0.5 prior to culture in normal (iron-replete) medium containing various doses of DFO or Bpdl for 24 h. Infectious progeny were harvested and replated onto fresh monolayers for quantification. *C. trachomatis* growing in iron-replete medium consistently produced over 10^7 IFUs/ml. No significant inhibition of recoverable IFUs was observed upon addition of low doses (25 or 50 µM) of Bpdl to the culture medium. The same doses of DFO (25 and 50 µM) each caused an approximate 70% decrease in IFU output that was significantly lower than the untreated control (unpaired, two-tailed *t*-test; $p = 0.0046$ and $p = 0.0072$, respectively). However, at concentrations of 100 and 250 µM, Bpdl was more effective relative to the untreated sample. At 100 µM, Bpdl caused a 2.5-log reduction of recoverable IFUs from the untreated control ($p = 0.0001$). Moreover, inhibition of infectious progeny

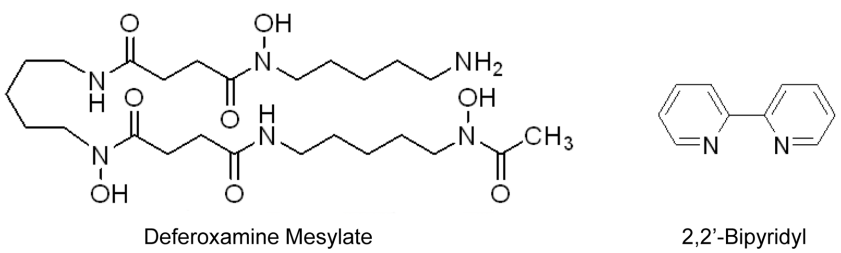

FIGURE 1 | Chemical structure of iron-chelating agents. Deferoxamine mesylate (DFO) is a hydrophilic compound with a high affinity for ferric iron (Martell and Smith, 1977). It binds ferric iron in a 1:1 (hexadentate) complex (Gaeta and Hider, 2005). 2,2′-bipyridyl (Bpdl) is a membrane permeable compound with similar moderate affinities for Fe^{2+} and Fe^{3+} (Smith and Martell, 1975), and binds both species of these molecules in a 3:1 molar ratio (bidentate; Gaeta and Hider, 2005).

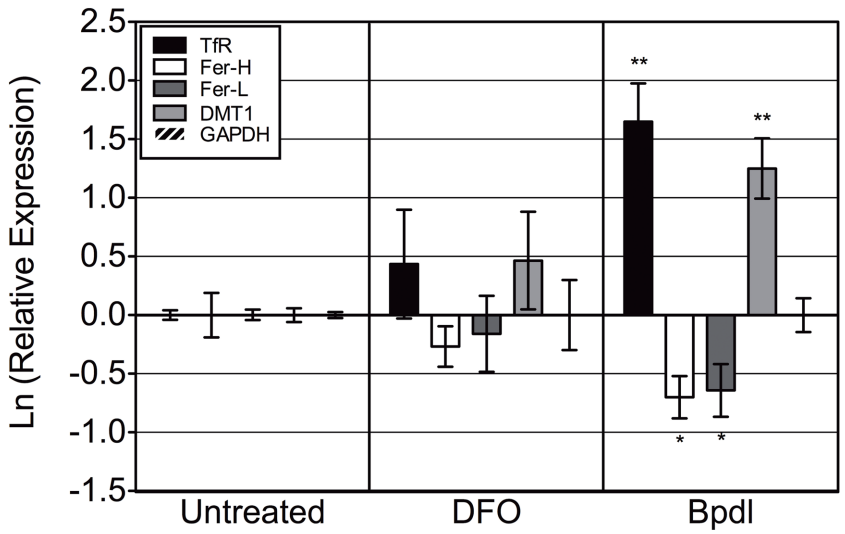

FIGURE 2 | Bipyridyl treatment modulates eukaryotic iron-responsive transcription. Human epithelial HEp2 cells were incubated in complete medium containing 100 μM DFO or BpdI for 10 h before select transcript levels were measured via RT-qPCR. Reported iron-responsive genes, transferrin receptor (TfR) and divalent metal transporter 1 (DMT1), and iron-repressed genes, ferritin heavy and light chain (Fer-H/L) were normalized to endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels and then calibrated to the untreated control (ΔΔCt method; Pfaffl, 2001). The *y*-axis presents the natural log of the target gene relative expression. Bars represent the natural log of mean relative expressions ± SD in one representative experiment containing two biological replicates per treatment, each assayed in triplicate. Experiment was performed independently two times and obtained similar transcriptional profiles in each case (i.e., total of four biological replicates were observed). Statistical significance from the untreated was determined by unpaired, one-tailed *t*-test (*$p < 0.05$, **$p < 0.01$).

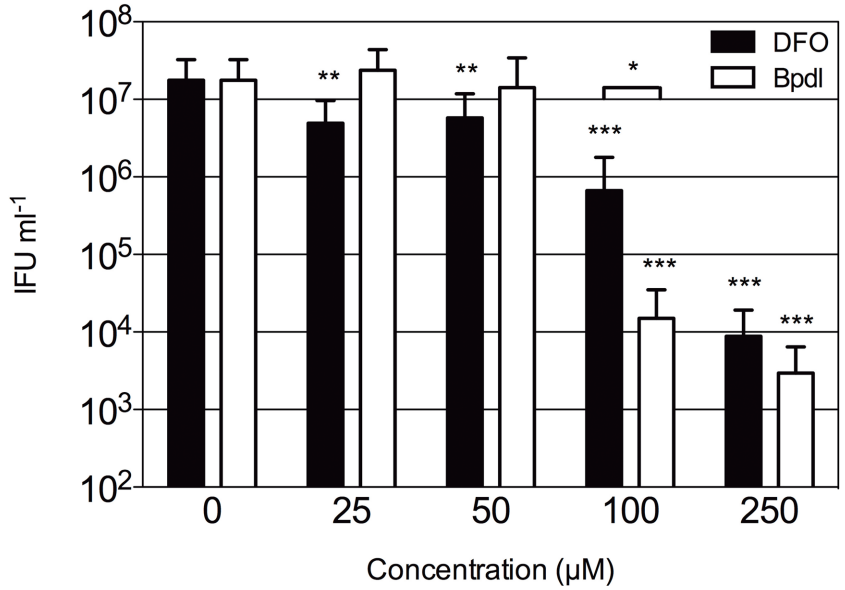

FIGURE 3 | Infectious progeny of *C. trachomatis* samples cultured with iron-chelating agents. Various concentrations of DFO (black bars) or BpdI (white bars) were included in the culture medium added immediately post-chlamydial invasion. Infectious titers were quantified after 24 h of treatment and presented as inclusion forming units (IFU) per milliliter of inoculum (*y*-axis). Graph represents the mean ± SD derived from at least two independent experiments, assayed in triplicate. Statistical significance determined with unpaired, two-tailed *t*-test in conjunction with *F*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Asterisks without brackets represent comparison to the untreated control; brackets indicate the comparison of DFO to BpdI at the same concentration.

was highly reproducible with the percent recovery ranging from just 0 to 0.33% of the untreated control over three independent experiments. At 100 μM concentration administered at the time of infection, DFO yielded a 95% reduction from the untreated control ($p = 0.0002$). However, this result was highly variable with a percent recovery that ranged from 0 (complete arrest) to 12.5% of the untreated control. Compared directly, treatment with BpdI was 26-fold more effective than DFO in limiting the development

of infectious progeny ($p = 0.0408$). While both compounds caused similar reduction in recoverable IFU at 250 μM, some cells were lost indicating possible cytotoxicity (data not shown). Therefore, 100 μM was deemed a suitable concentration for both iron-chelating agents for subsequent experiments.

As the loss of recoverable infectious progeny due to developmental arrest is a hallmark of the chlamydial persistent phenotype (Beatty et al., 1994), we examined *C. trachomatis* samples cultured with Bpdl for another characteristic of persistence, the formation of aberrantly enlarged RBs. Infected cell cultures were treated for 24 or 48 h with 100 μM concentrations of Bpdl or DFO and visualized by immunofluorescent confocal microscopy (**Figure 4**). Compared to the untreated control, culture with both DFO and Bpdl (100 μM, administered at the time of infection) caused a qualitatively similar reduction in the size of chlamydial inclusions at 24 h p.i. (**Figures 4A–C**). Enlarged, aberrant RBs, characteristic of the persistent phenotype, were only observed after 48 h of culture with Bpdl (**Figure 4F**). Interestingly, the same 48-h length of treatment with DFO (**Figure 4E**) yielded a population of chlamydial inclusions, the majority of which resembled that of the untreated, 24-h p.i. sample (**Figure 4A**), suggesting that DFO may not be suitable for time-points of this length. It should be noted that growth media was neither replaced nor additional chelators added, consistent with our intention of minimizing manipulation of the experimental system. Under such conditions, Bpdl appeared to be more efficient in initiating and maintaining a developmental arrest in *C. trachomatis*.

CHLAMYDIAL VIABILITY UPON BIPYRIDYL TREATMENT

The term "persistence," refers to the ability of non-cultivatable chlamydiae to re-enter the acute life-cycle and cause disease upon the removal of the condition of stress. We chose to examine the reversibility of the persistent phenotype of chlamydia exposed to Bpdl in two ways. First, persistent C*hlamydiae* often exhibit continued genomic replication, albeit at a reduced rate, despite a lack of re-differentiation into the infectious EB form (Belland et al., 2003; Lambden et al., 2006). Therefore *Chlamydia*-specific genomic DNA (gDNA) was monitored longitudinally during iron-chelation treatment using real time qPCR. Incubation with DFO and Bpdl (100 μM) retarded chlamydial DNA replication, compared to the untreated control. However, consistent with the recoverable IFU and IFA data, Bpdl treatment caused an even greater growth defect than that of DFO-treatment (**Figure 5A**). During the exponential phase of growth (between 12 and 24 h post-infection), Bpdl-treated *C. trachomatis* gDNA doubled once every ~5.5 h, compared to approximately each 2.5 and 3.3 h for untreated and DFO-treated cultures, respectively, over the same time period. Importantly, the number of genomes increased during each time period tested

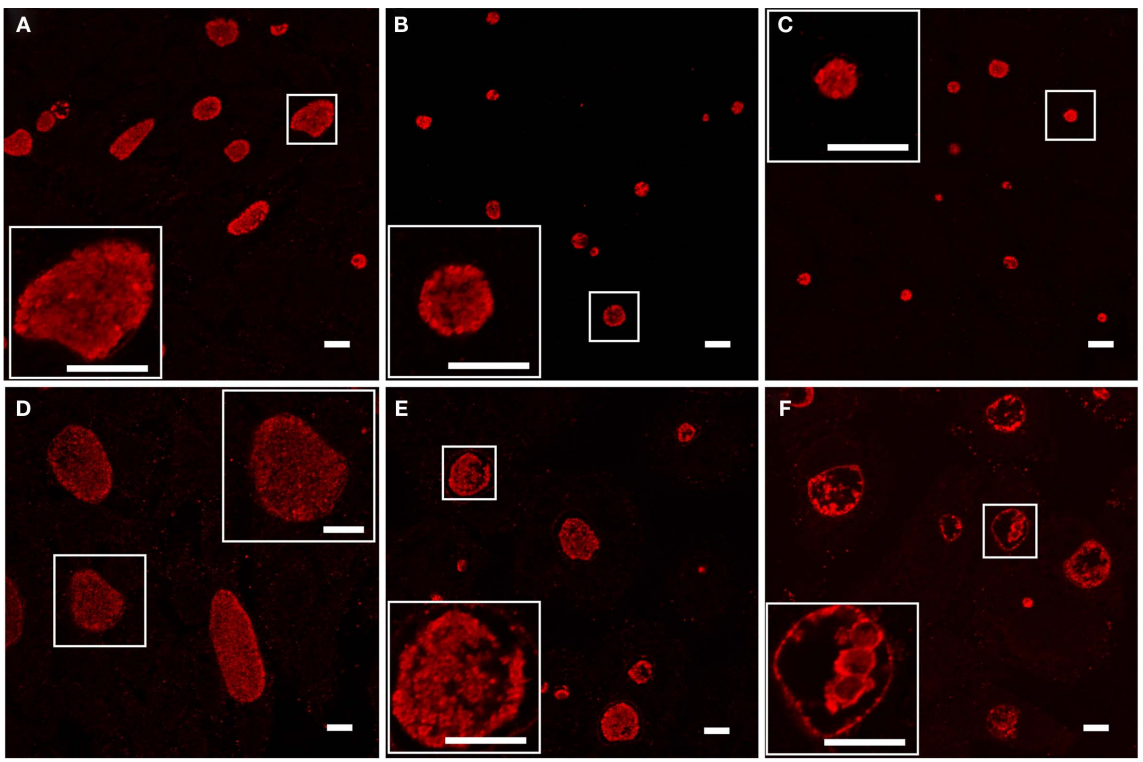

FIGURE 4 | Chlamydial morphology after long-term incubation with inhibitory doses of Deferoxamine or Bipyridyl. *C. trachomatis*-infected cell cultures were incubated with 100 μM DFO **(B, E)** or Bpdl **(C,F)** in conjunction with untreated controls **(A,D)**. Images from the top panel row **(A–C)** were fixed after 24 h p.i. and images from the bottom panel row **(D–F)** were fixed after 48 h p.i. Samples were stained immunofluorescently using convalescent human sera. Aberrant RB morphology and a lack of EB particles within the inclusional lumen was only observed upon 48 h of Bpdl treatment **(F)**. Samples treated with DFO for 48 h **(E)** exhibited similar characteristics of untreated 24 h growth **(A)**. Images were captured using Leica Confocal microscope. Bars represent 10 μM in each panel/inset. Images selected represent the overall trends observed in four independent experiments.

for Bpdl-cultured samples, indicating active genomic replication, and thus viable organisms, despite the lack of infectious EB re-differentiation.

Reactivation of developmentally arrested chlamydiae upon removal of the chelator was monitored using recoverable IFUs as a measure of viability. Infected HEp2 cells were incubated with Bpdl for 18 or 24 h, and then "reactivated" by the replacement of Bpdl-supplemented media with normal (iron-replete) medium for another 18 to 24 h of incubation. The infectious titer of each sample was quantified and compared to samples incubated with Bpdl for the entire course of infection (**Figure 5B**). A near complete arrest of infectious EB re-differentiation was observed at 24, 36, and 48 h of continuous treatment with Bpdl, which was consistent with the aberrant morphology observed by immunofluorescent analysis at 48 h p.i. (**Figure 4F**). In contrast, "reactivated" samples exhibited an increase in recoverable infectious progeny of 2–3 logs over continuously treated samples. Although reactivated chlamydial samples were not completely rescued to the untreated output levels, a majority of chlamydiae were able to re-enter the acute life-cycle and re-differentiate into the infectious EB form.

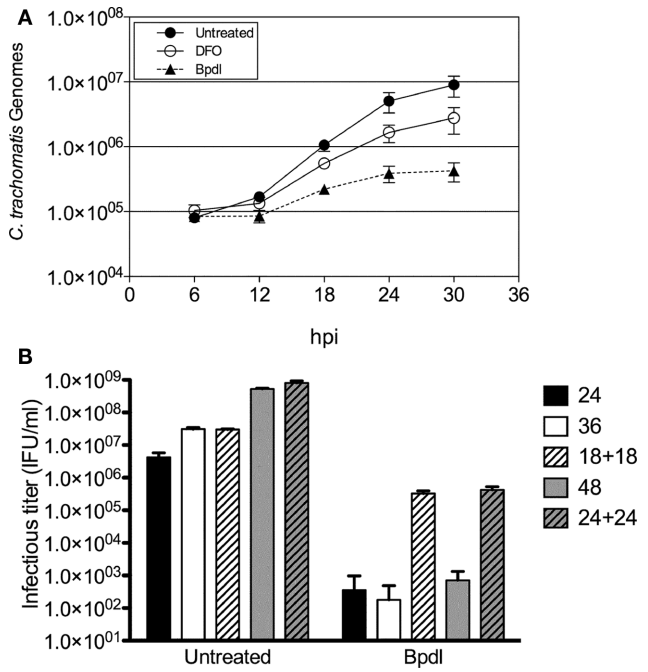

FIGURE 5 | Viability of *C. trachomatis* cultured with Bipyridyl. (A) Chlamydia-specific DNA was quantified via RT-qPCR from total genomic DNA extracted each 6 h. The numbers of chlamydial genomes (*y*-axis) were extrapolated from a standard curve of genomic DNA obtained from purified chlamydial EBs. Data points represent the mean ± SD of two independent experiments, which were representative of greater than five independent time course experiments. **(B)** Bpdl-cultured *C. trachomatis* were reactivated by washing and incubation with normal, iron-replete medium ("18 + 18" and "24 + 24") and compared to cultures treated with Bpdl throughout the entire experiment ("24," "36," "48"). *C. trachomatis* treated with Bpdl for 18 and 24 h were still viable when cultured with normal media. Graph represents data combined from two independent experiments, each assayed in triplicate. Bars represent mean ± SD.

EXOGENOUS IRON ABROGATES CHLAMYDIAL INHIBITION CAUSED BY BIPYRIDYL AND DEFEROXAMINE

To ensure that effects elicited by culture with Bpdl resulted from the restriction of iron, an iron-titration experiment was performed, in which increasing concentrations of Fe^{3+} or Fe^{2+} were added to an inhibitory dose (100 μM) of the two iron-chelating agents, prior to their incubation with infected cell cultures. To confirm the binding capabilities of Bpdl, we utilized species of both ferric ($FeCl_3$) and ferrous ($FeSO_4$) oxidative states of iron. Based on reported affinities (Smith and Martell, 1975; Martell and Smith, 1977), it was not surprising that both Bpdl and DFO-mediated inhibition was abrogated by the addition of exogenous ferric chloride (**Figure 6A**). In contrast, the addition of ferrous sulfate was not able to rescue DFO-mediated inhibition of recoverable IFU output (**Figure 6B**), which was expected, based on its reported specific affinity for ferric iron (Martell and Smith, 1977). The lack of recovery exhibited by ferrous iron supplementation illustrated the inability of DFO to bind the divalent metal in the extracellular medium, coupled with the inability of the host cell to efficiently import the supplemented ferrous iron. The end result was the unaltered sequestration of ferric iron in the extracellular media, which, of course, is the main source of host cell iron acquisition. This result was not the case for Bpdl-mediated inhibition, as recoverable IFU output was completely reversed at 20 μM of $FeSO_4$, which was the lowest dose tested, reflecting its ability to bind the divalent oxidation state

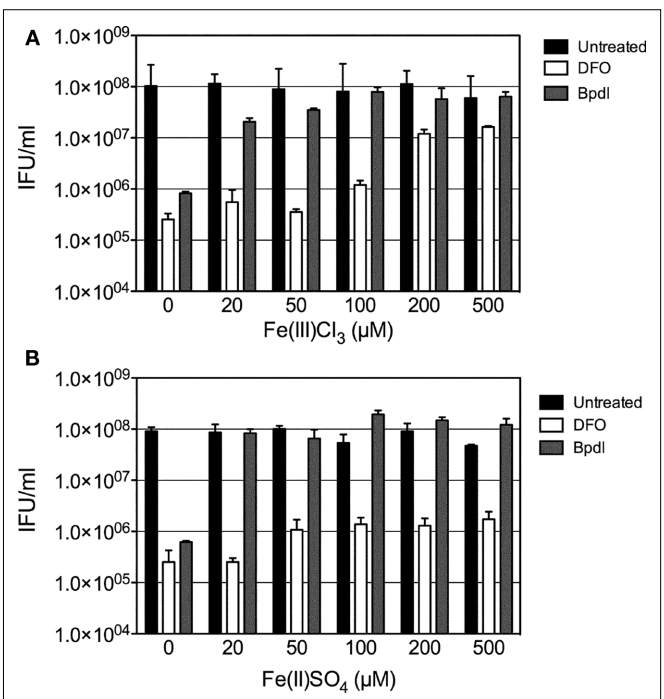

FIGURE 6 | The effect of iron on iron-chelator mediated recoverable IFU inhibition. Increasing concentrations of ferric **(A)** or ferrous **(B)** iron were used to titrate out the inhibition of *C. trachomatis* infectious progeny caused by treatment with (100 μM) Bpdl or DFO. Exogenous iron was added to medium containing chelators immediately prior to its use in culture. Samples were harvested at 24 h p.i. for infectious titer quantification. Bars represent the mean ± SD of one experiment representative of two independent trials in which similar trends were obtained.

of the metal. Taken together, the addition of exogenous ferric and ferrous iron abrogated the inhibitory effects of Bpdl, while only ferric iron restored IFU yield in DFO-treated samples.

THE EFFECT OF BIPYRIDYL TREATMENT ON CHLAMYDIAL TRANSCRIPTION

Having validated Bpdl treatment as an efficient mediator of the chlamydial persistent response, we decided to test its effects on chlamydial transcription of genes associated with persistence or known to be induced under iron-limiting conditions. We monitored the expression of known molecular markers of persistence – the continued expression of the early gene *euo* (Belland et al., 2003; Ouellette et al., 2006; Timms et al., 2009) and the failure to induce transcription of the late gene *omcB* (Iliffe-Lee and McClarty, 2000; Belland et al., 2003; Ouellette et al., 2006; Timms et al., 2009). Therefore the expression of persistence markers, *euo* and *omcB*, was measured longitudinally from DFO, Bpdl, and untreated cultures via RT-qPCR and normalized by the genome copy number derived

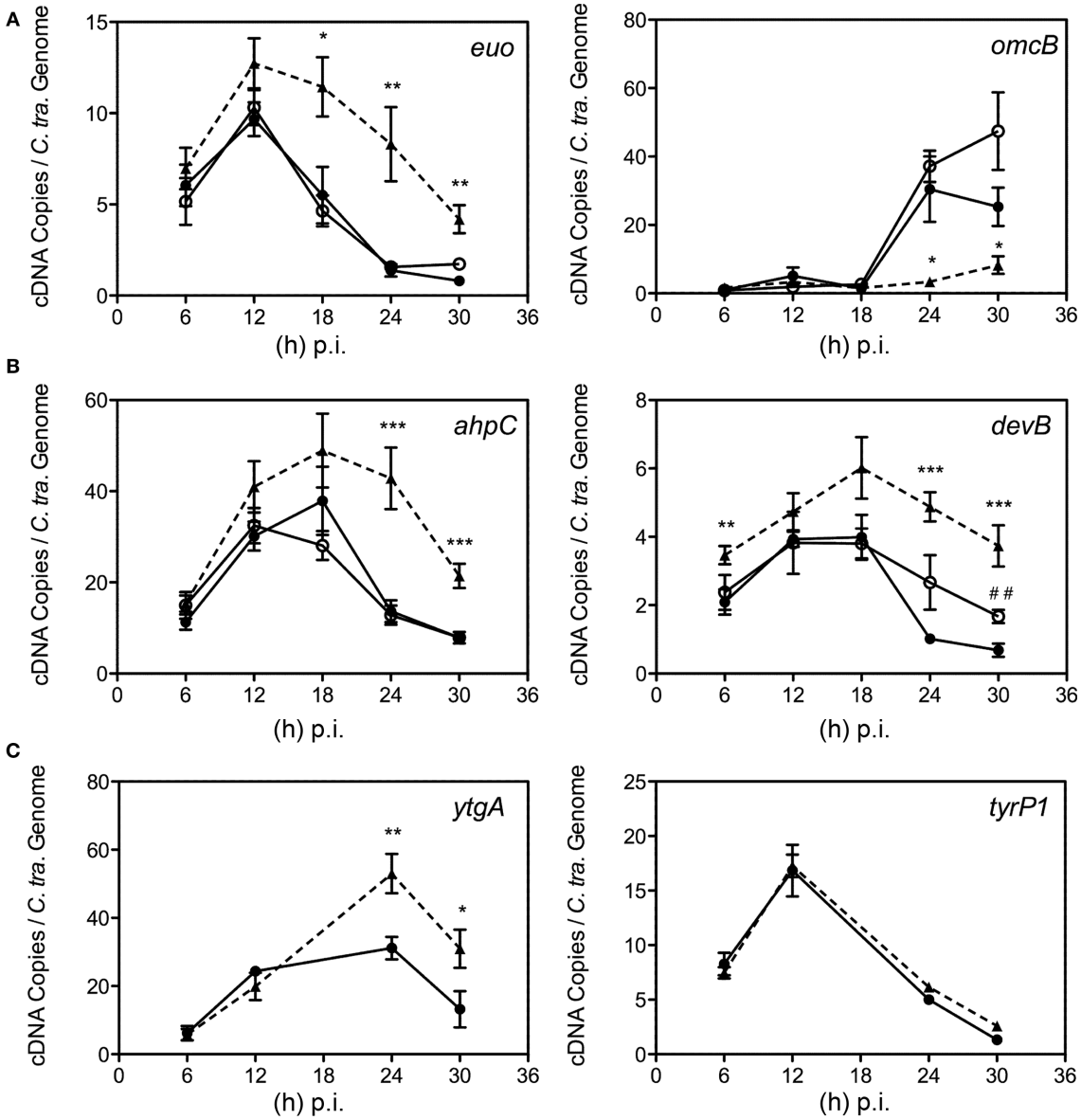

FIGURE 7 | Time course analysis of selected transcript expression of *C. trachomatis*. Samples were cultured in normal iron-replete medium (–•–), or in conjunction with deferoxamine (–o–) or bipyridyl (–Δ–). Total RNA was extracted longitudinally and reverse transcribed to cDNA for quantification using specific primer sets (**Table 1**). cDNA levels were adjusted to reflect the number of chlamydial organisms by using the number of chlamydial genomes determined from parallel samples. **(A)** Transcripts whose expression were altered under multiple models of persistence (Iliffe-Lee and McClarty, 2000; Belland et al., 2003; Ouellette et al., 2006; Timms et al., 2009). **(B)** Transcripts from *C. trachomatis* whose expression were altered under iron-restriction. **(C)** *ytgA*, a transcript whose expression was hypothesized to be responsive to fluctuations in iron availability, along with tyrP1 whose transcription was altered under IFN-g treatment, but not iron-restriction in *C. pneumoniae* (Timms et al., 2009). Statistical significance relative to the untreated control was determined using two-tailed *t*-test (Bpdl-treated samples: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; DFO-treated samples: $^{\#\#}p < 0.01$).

from a parallel sample. As expected, appreciable *omcB* expression was not observed until 18 to 24 h p.i. in the untreated control, which correlates to the onset of EB re-differentiation for *C. trachomatis*. Alternatively, the expression of *euo* in the same samples increased during the earliest time-points measured and then decreased to near absent levels during the late stages of infection (**Figure 7A**). Culture with 100 μM DFO from the time of infection did not alter either of these transcriptional profiles, suggesting that a persistent phenotype could not be induced efficiently by this iron chelator when administered at the time of infection. In contrast, incubation with Bpdl caused the extended repression of *omcB* transcript expression throughout the entire time course. Concomitantly, expression of *euo* remained elevated until the late stages of infection. The altered expression of these two genes indicated that culture with Bpdl (100 μM), from the time of infection, elicited a persistent chlamydial phenotype.

We sought to validate the Bpdl model by monitoring its effects on the transcription of two previously reported chlamydial iron-responsive genes, *ahpC* and *devB* (Dill et al., 2009), in parallel with untreated and DFO-treated cultures. When included immediately post-invasion, a 100-μM concentration of DFO, noticeable increases in the levels of *devB* transcript were only observed at 24 (2.6-fold) and 30 (2.5-fold) h p.i. (unpaired, two-tailed *t*-test; $p = 0.0740$, $p = 0.0023$, respectively), while no differential transcription of *ahpC* was observed (**Figure 7B**). In contrast, culture with Bpdl caused statistically significant elevation over the untreated control at multiple time-points of both *ahpC* and *devB* (**Figure 7B**), with a statistically significant increase observed as early as 6 h post-treatment for *devB*. Maximum differential expression occurred at 24 h p.i. for *ahpC*, and reached 3.1-fold over the untreated levels ($p = 0.0008$). For *devB*, maximum differential expression reached 5.6-fold at 30 h p.i. ($p = 0.0009$). Taken together, treatment with Bpdl caused a more robust induction *ahpC* and *devB* than treatment with DFO. Not only was the altered expression more pronounced, it also lasted longer, suggesting significant and sustained iron-deprivation.

We also analyzed the expression of *ytgA*. YtgA, a proposed periplasmic iron-binding protein, was reported to be iron-responsive at the protein level upon long-term treatment with DFO (Miller et al., 2009), yet its differential expression at the transcript level has never been shown, and thus served as an excellent candidate to further evaluate the efficiency of iron starvation mediated by Bpdl. Using the Bpdl model, expression of *ytgA* was clearly elevated after 24 and 30 h p.i. (unpaired, two-tailed *t*-test; $p = 0.0083$, $p = 0.0433$, respectively; **Figure 7C**), providing a mechanism for the elevated protein expression previously observed (Miller et al., 2009). Thus, the greater effectiveness of the new iron starvation method was validated by the discovery of *ytgA* being an iron-responsive gene at the level of transcription.

To ensure that the up-regulation of *ahpC*, *devB,* and *ytgA* were specific to iron-limitation, the expression of *tyrP-1,* which codes for an aromatic amino acid transporter, was monitored. Its transcript expression in *C. pneumoniae* was differentially expressed under persistence mediated by IFN-g treatment, but not iron-restriction (Timms et al., 2009). IFN-g is thought to induce expression of the host enzyme, indoleamine dioxygenase (IDO), which effectively limits the available amount of tryptophan within the cell (Wood et al., 2004). Therefore its elevated expression under IFN-g mediated persistence presumably functions to increase acquisition of the limited amino acid. Because Timms et al. (2009) reported that it was not differentially expressed under conditions of low-iron availability, its transcript expression was monitored to distinguish the effects of Bpdl-mediated iron starvation from general persistence. As expected, transcription of *tyrP1* was not altered under Bpdl treatment at any time-point tested (**Figure 7C**). The fact that this mid-stage gene was not differentially expressed under Bpdl-mediated persistence indicated that the elevated transcription published in this study was specific to iron-restriction. Taken together, the data indicate collectively that a single-dose Bpdl treatment is an efficient iron-restriction model for the growth of *C. trachomatis*, and is able to elicit a more uniform, and consequently, a more robust response from *Chlamydiae* than DFO under identical treatment conditions.

DISCUSSION

We present a re-evaluation of the currently available iron starvation protocol for intracellular bacteria by directly comparing DFO and Bpdl for their abilities to induce iron starvation-related phenotypes in the obligate intracellular pathogen *C. trachomatis*. The goal was to identify an alternative means of starving *Chlamydia* of iron with minimal manipulations, such as pre-starving the host cell, or preventing it from synthesizing proteins involved in iron acquisition. Our motivation for simplifying iron starvation of *Chlamydia* by excluding such manipulations was to minimize potential contributory factors that may confound data interpretation. To this end, we identified Bpdl to be a better iron chelator than DFO under single-treatment experimental conditions. In a number of criteria, specifically IFU yield, aberrant RB formation, and transcriptional modulation of previously reported persistence indicating genes, Bpdl was consistently more potent than DFO. Moreover, the increased differential transcription of iron-responsive genes, *ahpC* and *devB,* in the Bpdl protocol relative to the DFO starvation method validated the alternative iron starvation system. Finally, the Bpdl model allowed the novel observation that *ytgA*, which codes for an iron-binding periplasmic protein thought to be involved in iron acquisition/transport into *Chlamydia,* is iron-responsive at the transcript level. The use of this Bpdl model could allow additional insights into the global iron-restricted regulon of *C. trachomatis*.

Our study was initiated due to the inefficient ability of DFO to elicit a uniform, persistent phenotype of *C. trachomatis* for analysis of differential gene expression. We hypothesized that DFO, which is not membrane permeable and can only bind ferric iron, could only be efficient in the iron-restriction of the obligate intracellular pathogen if the only chlamydial source of iron was the direct acquisition of ferric iron as it entered the host cell via the transferrin pathway. On the other hand, if *Chlamydia* were able to acquire iron from another source, e.g., from the cytosolic labile iron pool or the slow recycling pathway (Ouellette and Carabeo, 2010), then use of DFO would require longer treatment to deplete intracellular iron stores. In the literature, DFO has rarely been used without auxiliary steps or methods for iron-restriction. For instance, the combination of DFO with cyclohexamide (which is a general eukaryotic protein synthesis inhibitor) has been employed to prevent the host cell up-regulation of the transferrin iron-acquisition pathway (Raulston, 1997; Wehrl et al., 2004; Dill et al., 2009). Yet the

modulation of host protein synthesis results in pleiotropic effects that likely influence chlamydial growth via means independent of iron starvation. Others have eliminated this need for cyclohexamide through the use of long-term DFO pre-treatment protocols in order to deplete host cells of intracellular iron stores prior to chlamydial invasion (Al-Younes et al., 2001; Peters et al., 2005; Mukhopadhyay et al., 2006; Dill et al., 2009; Timms et al., 2009). While the effects of this protocol could presumably be attributed solely to iron-deprivation, it is tedious and requires the infection of a compromised host cell population. One particularly illustrative example was published by Dill et al. (2009), in which both pre-treatment and combination with cyclohexamide was employed to observe transient and marginal (less than twofold) elevation of a handful of iron-responsive *C. trachomatis* transcripts. Meanwhile, transcripts of *euo* and *omcB* were not modulated (Dill et al., 2009), suggesting that the chlamydial persistent phenotype was not elicited by this treatment combination.

The salient properties of Bpdl are its membrane permeability and its high affinity for the ferric and ferrous forms of iron. Membrane permeability allows it access to the different iron reservoir inside the cell, as well as within the chlamydia organisms, while its ability to bind both forms of iron would result in a more complete iron depletion. Both properties combine to rapidly and efficiently starve chlamydia and its host of iron. An iron chelator with similar properties, salicylaldehyde isonicotinoyl hydrazone (SIH) was initially the compound of choice. However, its structural similarity to a family of molecules known to inhibit the function of type III secretion apparatus in a number of bacterial pathogens precluded accurate interpretations of its growth effects on chlamydia. Indeed, we have observed the inhibition of the cytosolic translocation of the *Salmonella* effector PipB2 in infected cells treated with SIH for 8 h (Thompson and Carabeo, unpublished data), and hence, investigations of the functions of type III secretion in *Chlamydia* using these SIH-related compounds must be taken with caution.

In summary, the detailed characterization of DFO and Bpdl with regards to their effects on *C. trachomatis* when supplemented at the time of infection, revealed the superiority of Bpdl. Moreover, this model is easier to apply and eliminated many of the caveats of other DFO- based iron-restriction protocols. As such, efforts to identify *bona fide* primary iron-responsive regulons may now be compatible with microarray approaches, given the availability of a faster acting iron chelator that, in addition to the extracellular iron pool, targets the pools in the DFO-inaccessible cytosolic, organellar, and chlamydial environment.

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The chlamydial type III secretion mechanism: revealing cracks in a tough nut

Helen Jennifer Betts-Hampikian and Kenneth A. Fields*

Department of Microbiology and Immunology, University of Miami Miller School of Medicine, Miami, FL, USA

Edited by:
Rey Carabeo, Imperial College London, UK

Reviewed by:
Richard Hayward, University College London, UK
Marci Scidmore, Cornell University, USA

***Correspondence:**
Kenneth A. Fields, Department of Microbiology and Immunology, University of Miami Miller School of Medicine, 1600 NW 10th Avenue, Miami, FL 33136, USA.
e-mail: kfields@med.miami.edu

Present-day members of the Chlamydiaceae contain parasitic bacteria that have been co-evolving with their eukaryotic hosts over hundreds of millions of years. Likewise, a type III secretion system encoded within all genomes has been refined to complement the unique obligate intracellular niche colonized so successfully by *Chlamydia* spp. All this adaptation has occurred in the apparent absence of the horizontal gene transfer responsible for creating the wide range of diversity in other Gram-negative, type III-expressing bacteria. The result is a system that is, in many ways, uniquely chlamydial. A critical mass of information has been amassed that sheds significant light on how the chlamydial secretion system functions and contributes to an obligate intracellular lifestyle. Although the overall mechanism is certainly similar to homologous systems, an image has emerged where the chlamydial secretion system is essential for both survival and virulence. Numerous apparent differences, some subtle and some profound, differentiate chlamydial type III secretion from others. Herein, we provide a comprehensive review of the current state of knowledge regarding the *Chlamydia* type III secretion mechanism. We focus on the aspects that are distinctly chlamydial and comment on how this important system influences chlamydial pathogenesis. Gaining a grasp on this fascinating system has been challenging in the absence of a tractable genetic system. However, the surface of this tough nut has been scored and the future promises to be fruitful and revealing.

Keywords: secretion, virulence, intracellular

INTRODUCTION

The Family Chlamydiaceae contains obligate intracellular, Gram-negative bacteria capable of infecting an impressive array of hosts including reptiles, amphibians, and animals. Three species, *C. trachomatis*, *C. pneumoniae*, and the zoonotically acquired *C. psittaci*, are significantly associated with human disease. Ocular (serovars A–C) or genital (serovars D–K, L1–L3) infections with *C. trachomatis* represent leading causes of infectious blindness and sexually transmitted disease, respectively. *C. pneumoniae* is a common respiratory pathogen associated with relatively mild, community-acquired pneumonias (Kuo et al., 1995), whereas *C. psittaci* respiratory infections, acquired rarely from an avian reservoir, cause an atypical pneumonia which can be fatal (Sessa et al., 2009). The prevalence of *Chlamydia*-mediated human disease, the overall broad host range, and the identification of environmental *Chlamydia*-like organisms has led to the assertion that the Chlamydiales represent a group of successful parasites that are virtually ubiquitous in nature.

Indeed, considerable effort has been invested to elucidate molecular mechanisms that contribute to this overall success. As obligate intracellular pathogens expressing a minimal genome, many distinct factors likely orchestrate an intricate and highly interdependent manipulation of host cell biology to culminate in successful pathogenesis. The type III secretion system (T3SS) has emerged as one mechanism capable of promoting chlamydial virulence. Genes encoding the virulence-associated T3SS were first identified in *Yersinia* spp. (Hueck, 1998) and subsequent characterization of the archetype Ysc-Yop system revealed an intricate bacterial mechanism to subvert eukaryotic hosts. T3SSs have since been identified in a wide variety of medically significant Gram-negative pathogens where they are essential for virulence (He et al., 2004). Intriguingly, the T3SS has also been shown to mediate symbiotic relationships and more recently, several non-pathogenic organisms have been found to contain T3SS genes (Krishnan et al., 2003; Tampakaki, 2004; Pallen et al., 2005). We will employ the term "non-flagellar T3SS (NF-T3SS)" throughout this review to differentiate from the process of flagellar biogenesis and in reference to the broader capacity of T3S to mediate interactions between bacteria and a respective eukaryotic host cell (Pallen et al., 2005).

The NF-T3SS is often referred to as acting like a "molecular syringe" to deliver anti-host bacterial "effector" proteins directly into a host cell in a contiguous process (Hueck, 1998). Essential components of the NF-T3SS (**Figure 1**) include (i) ancillary components (AC) functioning within the bacterial cytoplasm and represented by a set of chaperones and regulatory factors, (ii) membrane-associated gene products that form a multipartite core secretory apparatus that is highly conserved among systems, (iii) proteins comprising an extended needle and tip complex (NC and TC, respectively) which bridges the space between bacterium and host membranes, and (iv) secreted translocon (Tr) proteins required to form pores in eukaryotic membranes through which secretion substrates gain access to the host cytosol (Ghosh, 2004; Tampakaki, 2004). Collectively referred to as the "injectisome", secretion activity of these machines is tightly regulated and stimulated only upon contact with target host molecules. The reader is referred to several recent reviews for a detailed overview of the T3S mechanism in other bacteria (Moraes et al., 2008; Mueller et al., 2008; Marlovits and Stebbins, 2009; Beeckman and Vanrompay, 2010).

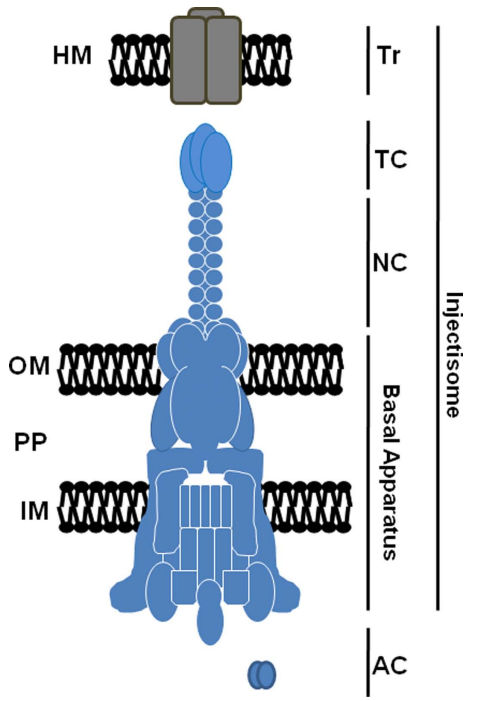

FIGURE 1 | Schematic representation of a prototypical T3S injectisome. Components including the translocator (Tr), tip (TC), and needle (NC) complexes, basal apparatus, and cytoplasmic ancillary proteins (AC) are shown. The basal apparatus spans the bacterial inner membrane (IM), periplasm (PP), and outer membrane (OM). Secreted translocon components are shown localized to a host membrane (HM). Correct stoichiometry of multimeric proteins is not indicated.

In 1997, Hsia et al. identified a locus of four open reading frames (ORFs) in *C. psittaci* with homology to *Yersinia* T3S proteins YscU, LcrD/YscV, YopN, and SycE respectively (Hsia et al., 1997). Initial genome sequencing projects then confirmed an entire complement of conserved, putative T3SS genes in *C. trachomatis* serovar D and *C. pneumoniae* (Stephens et al., 1998; Kalman et al., 1999). Genome sequencing projects have subsequently identified ORFs capable of encoding a complete T3SS in all sequenced members of the *Chlamydiaceae* family (Read et al., 2000, 2003; Thomson et al., 2005; Azuma et al., 2006) and more recently *Chlamydia*-related bacteria infecting amoebae (Bertelli et al., 2010). The T3S mechanism is therefore clearly entrenched deeply in the biology of these fascinating bacteria. As expected, questions regarding how the T3SS functions and contributes to chlamydial survival have been slow to find answers. This is largely due to the genetic intractability of *Chlamydia* spp. and a complex parasitic developmental cycle. In recent years, however, there has been a significant increase in our understanding of the role of the NF-T3SS in chlamydial pathogenesis and development. Like other NF-T3SSs, the basic secretion mechanism is likely conserved. Yet variations, consistent with *Chlamydia*'s unique biology, have been noted. Certainly, the emerging complement of niche-specific, secreted effector proteins provides a significant basis for distinctive biology. In fact, several recent reviews provide excellent overviews of chlamydial effector proteins deployed by the T3S mechanism (Valdivia, 2008; Betts et al., 2009; Cocchiaro and Valdivia, 2009). In this review, we will focus on the chlamydial T3S apparatus and comprehensively examine current knowledge regarding the mechanics of this remarkable nanomachine in *Chlamydia* spp. Rather than focus solely on how the chlamydial T3SS is the same as prototypical systems, we will emphasize variations found in *Chlamydia* (summarized in **Table 1**). We endeavor herein to consider how these variable attributes respond to, govern, or otherwise contribute to the successful obligate intracellular lifestyle exemplified by *Chlamydia* spp.

A thorough consideration of the chlamydial T3SS cannot proceed without comment on key aspects of chlamydial physiology and development. For example, *Chlamydia* spp. possess an atypical peptidoglycan layer (McCoy et al., 2006). Osmotic stability of infectious particles is instead likely conferred predominantly by disulfide bonding of cysteine-rich outer membrane (OM) and periplasmic proteins (Hatch, 1996). Therefore, the chlamydial T3SS need not contend with remodeling a dense peptidoglycan layer but must accommodate a disulfide linked envelope that is reduced during vegetative growth and oxidized in infectious particles. All *Chlamydia* spp. exhibit a unique bi-phasic developmental cycle which occurs within parasitophorous membrane bound vesicles, termed inclusions. Development is manifested when infectious, metabolically inert elementary bodies (EBs) invade epithelial cells and begin to differentiate into vegetative, non-infectious, metabolically active reticulate bodies (RBs). RBs replicate within the expanding inclusion before they differentiate back into EBs that are subsequently released, completing the developmental cycle (Abdelrahman and Belland, 2005). As emphasized below, the chlamydial T3SS is present and functional at each stage of this complex cycle.

The unique physiology noted above results in salient and subtle differences between the chlamydial T3S machinery and other systems. In the following section we will discuss characterized components of the chlamydial T3SS and, where appropriate, speculate on latent candidates. A T3SS-specific nomenclature based on the characterized *Yersinia* system was suggested by Hsia et al. (1997) where components of the basal apparatus are denoted with contact-dependent secretion (Cds), secreted or mobile components as *Chlamydia* outer protein (Cop), and cytoplasmic chaperones as specific *Chlamydia* chaperone (Scc). We will continue to use this nomenclature herein. To maintain conciseness, only designations found in the *C. trachomatis* serovar D genome database[1] will be employed when function is ambiguous or names have not been formally assigned.

ORIGIN AND FUNCTION OF THE CHLAMYDIAL T3SS

Generally, genes encoding components of the T3SS apparatus are clustered together in pathogenicity islands (PAIs) on a plasmid or the bacterial chromosome. Nucleotide content of T3SS coding sequences typically differ from that of the overall genome and are often denoted by a low G+C ratio (Tampakaki, 2004). In contrast, chlamydial genome analyses indicate a homogenous G+C content for T3SS-encoding genes that is similar to the roughly 40% level found in the remainder of respective genomes (Stephens et al., 1998). Moreover, T3SS genes are disseminated similarly throughout chlamydial genomes in four main clusters (Fields and Hackstadt, 2006) arranged into multiple operons (Hefty and Stephens, 2007). Although some of the clusters

[1]http://stdgen.northwestern.edu/

Table 1 | Distinguishing features of *Chlamydia* T3SS components[a].

Chlamydial T3SS component[b]	*Yersinia* counterpart	Putative function	*Chlamydia*-specific properties
CHAPERONES AND REGULATORY			
Scc2 (CT576)	SycD	Class II chaperone for CopB	Basic PI, unique N-terminal consisting of 60 residues which do not share homology with other proteins in the data base or Scc3
Scc3 (CT862)	SycD	Class II chaperone CopB2	Neutral PI, unique 60 residue N-terminal region containing a canonical amphipathic helix absent in Scc2. Interacts with C-terminal domain of CopN in addition to CopB2
CT274	SycD?	Putative Class II chaperone by secondary structure analysis, interacts with CT161 and CT668	"Stand alone" ORF[c]. Putative Class II TPR containing chaperone interacts with putative T3S effectors.
CdsO (CT670)	YscO	Chaperone for putative molecular ruler, CdsP	May interact with CdsN in *C. pneumoniae*.
Mscs (CT260)		Multi-cargo effector chaperone, docks with inner membrane component CdsQ to presumably facilitate delivery of effectors to CdsN for secretion.	"Stand alone" ORF. Able to bind CdsQ directly leading to speculation that Mscs dimers may be pre-docked at the cytoplasmic portion of the inner membrane C-ring.
CT663	SycH?	Putative Class I chaperone, may be involved in negative regulation	Class I chaperone able to exert negative regulatory effect on σ^{66}-dependent transcription.
CT043		Putative Class I chaperone	"Stand alone" ORF
CopN (CT089)	YopN	T3SS "Plug". Involved in regulation of secretion by maintaining the secretion system in an inactive state prior to the presence of an inductive signal.	Required to support intracellular growth of *C. pneumoniae in vitro*. Interacts CdsN.
SECRETION APPARATUS			
CdsL (CT561)	YscL	Basal apparatus component interacts with CdsQ. Based on homology likely to be involved in regulation of CdsN by inhibiting ATPase activity. May also be involved in assembly of the apparatus	Capable of interacting with the C-terminus of CdsD. Also interacts with FliI and FlhA homologues in the Bacterial 2-hybrid system and pull-down assays
CdsN (CT669)	YscN	ATPase involved in release of effectors from cognate chaperones and unfolding effectors to render them secretion competent	Capable of binding with CdsD
CdsQ (CT672)	YscQ	Cytoplasmic C-ring component of apparatus, docks chaperone-effector complexes and presumably facilitates effector delivery to CdsN.	Interacts with multiple apparatus components including "flagellar homologs"
CdsD (CT664)	YscD	Likely forms the inner membrane ring with CdsJ	Has a unique N-terminus containing an additional FHA domain which along with its FHA-2 domain undergoes phosphorylation by cytosolic PknD *in vitro*. Capable of interacting with a novel subset of inner membrane proteins.
CdsC (CT674)	YscC	Outer membrane secretin	Large unique N-terminal region comprising a distinctive hydrophilic domain that is variable between *Chlamydia* spp. Lacks an obvious lipoprotein pilot required in other NF-T3SSs for secretin oligomerization and membrane association.
TRANSLOCATION MACHINERY			
CdsF (CT666)	YscF	Needle subunit protein	Contains two cysteine residues at the N-terminus
CT584	LcrV	Putative needle tip protein, may be involved in host-cell sensing and contact dependent activation of the secretion system	"Stand alone" ORF. Not recognized by human serum from *Chlamydia* infected patients
CopB2 (CT861)	YopB	Possible additional translocator	Present in the host-cell cytosol

[a]Only components that have features that clearly distinguish them from homologs in other T3SSs are listed.
[b]Component genomic designations are indicated based on the annotated genome of C. trachomatis serovar D (Stephens, 1998).
[c]"Stand alone" ORF designation indicates the respective gene is encoded outside of T3SS-associated loci.

are flanked by tRNAs, insertion elements, and gene arrangements consistent with horizontal gene transfers or PAIs are lacking. These observations have provided the basis for hypotheses proposing that the NF-T3SS found in *Chlamydia* spp. represents an ancestral or primordial system (Stephens et al., 1998; Kim, 2001). This notion is supported by the identification of T3S genes in the amoeba symbiont *Parachlamydia* sp. UWE25, indicating that T3SS genes were present when symbiotic and pathogenic *Chlamydia* diverged some 700 million years ago (Horn et al., 2004; Pallen et al., 2005). Chlamydial genomes also contain several T3S components having more robust similarity to the flagellar T3SS, raising the possibility that chlamydiae represent a missing link in the transformation of flagellar to non-flagellar T3SSs (Kim, 2001). However, multiple groups have since made compelling arguments that flagellar and non-flagellar systems evolved divergently from a common ancestor (Gophna et al., 2003; Pallen et al., 2005). In addition, it was noted that similar to *Chlamydia* spp., genes encoding one of the NF-T3SSs found in the proteobacterium *Chromobacterium violaceum* also have G + C content similar to the remaining genome and some apparatus components are encoded in a separated loci (Betts et al., 2004). Therefore, the evolutionary history of T3SSs remains clouded. Regardless of precise lineage, it is clear that the chlamydial T3SS has evolved in isolation for an evolutionarily significant span of time. This is in agreement with phylogenetic analyses that typically sort the chlamydial T3SS to a distinct family (Troisfontaines and Cornelis, 2005). One can therefore anticipate a great deal of T3S biology that is distinctly chlamydial.

Functionality of the chlamydial T3SS was first demonstrated indirectly. The chlamydial homolog of *Yersinia* YopN termed CopN was secreted by a heterologous T3SS when ectopically expressed in *Yersinia*, and immunolocalization data indicated secretion of the endogenous protein during chlamydial infection (Fields and Hackstadt, 2000). It is now doubtless that the primary function of the chlamydial T3SS is to translocate effector proteins into host cells for manipulation of eukaryotic cellular processes. Subsequent studies have further exploited the use of heterologous secretion systems to demonstrate an ability to secrete effector proteins (Subtil et al., 2001; Fields et al., 2003; Lugert et al., 2004; Ho and Starnbach, 2005). Furthermore, the development of small molecule inhibitors of T3S has now enabled more direct demonstration of T3SS-mediated secretion of endogenous chlamydial proteins (Nordfelth et al., 2005; Muschiol et al., 2006; Wolf et al., 2006; Bailey et al., 2007). We have speculated that *C. trachomatis* may secrete as many as 80 anti-host proteins (Betts et al., 2009). This number would be in line with an absolute dependence on host cell biology and underscores the importance of T3S in chlamydial survival. Combined with 20–30 apparent and predicted apparatus components, this T3SS gene content would represent roughly 10% of the 894 ORFs predicted within the *C. trachomatis* serovar D genome. This level of genomic commitment raises the possibility that functions beyond secretion of anti-host effector proteins likely exist for the chlamydial T3S apparatus. Several possibilities where the apparatus itself could contribute directly to chlamydial pathogenesis will be elaborated on below.

ASSEMBLING THE T3SS

THE APPARATUS SCAFFOLD

Current evidence from genetically tractable systems indicate that apparatus assembly is initiated by three proteins: an OM ring protein, a lipoprotein forming the inner membrane (IM) ring, and an IM-associated protein connecting the two rings (Yip et al., 2005; Spreter et al., 2009; Diepold et al., 2010). Based on sequence similarity, these proteins would correspond to chlamydial CdsC, CdsJ, and CdsD, respectively (**Figure 2**). It is likely that a similar outside–in

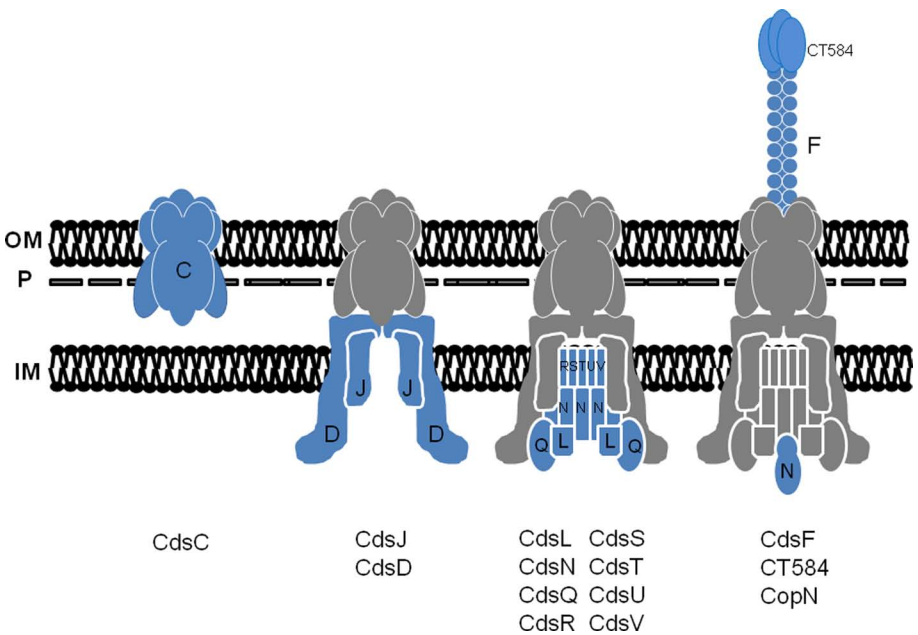

FIGURE 2 | A working model for the temporal assembly and composition of the chlamydial T3S apparatus. Stepwise addition of proteins is indicated with newly added components shown in blue and previously assembled components in gray (shown with Cds letter designation only). The basal apparatus spans the chlamydial inner membrane (IM), OmcB-containing P-layer (P), and outer membrane (OM). Flagellar homologs are omitted for clarity.

assembly is conserved in *Chlamydia* spp. Similar to their homologs, CdsC and CdsJ possess predicted Sec-dependent secretion signals that would be necessary for membrane access prior to injectisome assembly. Moreover, both CdsC and CdsJ partition as integral membrane proteins after Triton X-114 extraction of EBs (Fields et al., 2003). As predicted, CdsC is detectible in chlamydial OM complexes (Betts et al., 2008; Birkelund et al., 2009; Liu et al., 2010), whereas most of CdsJ is absent from this fraction (Betts et al., 2008). Aside from modeling predictions that indicate CdsJ is a lipoprotein, direct characterization of this protein is yet forthcoming. Consideration of CdsC and CdsD, however, reveals potentially interesting insights into the chlamydial system.

Based on sequence similarity, CdsC is a member of the PulD family of OM secretins (Hueck, 1998). Interestingly, primary sequence analysis reveals that the N-terminal 250 residues of CdsC comprise a distinctive hydrophilic domain that is unique to chlamydial CdsC yet divergent in primary sequence among chlamydial species. What additional function could be conveyed by this domain remains unclear as no significant homologies are detected via PSI-BLAST. Direct evidence for the predicted multimerization is lacking, and a lipoprotein pilot commonly required for other secretins to oligomerize and become membrane associated (Hueck, 1998) has not been identified for *Chlamydia* spp. This is perhaps not surprising given that pilot proteins typically display <15% sequence similarity among NF-T3SSs (Okon et al., 2008). However, structures formed by homologs of CdsC are similar in morphology to the multimeric, "rosette-like" structures first observed on *C. psittaci* EBs (Matsumoto, 1982b). Other candidates such as *C. trachomatis* PmpD have been proposed for these structures (Crane et al., 2006), yet it seems equally reasonable that they will correspond to the T3SS apparatus. Biochemical extraction data indicate that portions of CdsC are almost certainly surface-exposed (Betts et al., 2008) and Peters et al., (2007) pointed out that, in addition to looking very much like other T3SS rings in electron micrographs, the apparent inner diameter of the rosettes is similar to those of OM rings in *Yersinia* and *Salmonella*.

Chlamydial CdsD represents a novel member of the YscD family of proteins, which in *Yersinia* forms the IM ring in conjunction with YscJ (Silvia-Herzog et al., 2008; Diepold et al., 2010). Interestingly, only the ca. 400 C-terminal residues of CdsD contain regions of similarity to members of the YscD family including putative transmembrane and phospholipid binding (BON) domains (Johnson et al., 2008). YscD family proteins also contain an forkhead associated (FHA)-like domain at the N-terminus (Pallen et al., 2005). FHA domains are often involved in mediating phosphorylation dependent protein-protein interactions via phospho-serine or phospho-threonine peptides (Durocher et al., 2000). CdsD contains two predicted FHA domains, FHA-1 within the first 100 residues and FHA-2 within the C-terminal region that contains YscD similarity. It should be pointed out that early studies suggested that CdsD may be surface exposed on EBs or even secreted during infection (Tanzer and Hatch, 2001; Herrmann et al., 2006). However, the weight of evidence now casts doubt on those interpretations. For example, protein interaction studies (**Figure 3**) have provided evidence for the C-terminus of CdsD interacting with IM-associated T3S proteins CdsL, CdsQ (Johnson et al., 2008), and CdsN (Stone et al., 2008). These data would be consistent with at least some portion of CdsD being exposed to the chlamydial cytoplasm. More direct evidence for this exposure is apparent since both FHA domains have recently been shown (Johnson and Mahony, 2007) *in vitro* to be substrates for protein kinasae D (PknD). PknD is one of two currently identified chlamydial eukaryotic-like, IM-associated serine/threonine protein kinases (STPK) having confirmed activity (Verma and Maurelli, 2003). Moreover, PknD may play a role in chlamydial replication (Johnson et al., 2009). Unfortunately technical obstacles have so far precluded corroboration of CdsD phosphorylation *in vivo* so it is unclear how this may correspond to T3SS activity. Regardless, CdsD represents the first example of a T3SS apparatus protein that has been shown to undergo phosphorylation (Virok et al., 2005; Johnson and Mahony, 2007; Johnson et al., 2009). Considerable questions remain regarding CdsD contribution to chlamydial T3S. Although a single transmembrane domain is predicted, Triton X-114 solubility is inconsistent with CdsD being an integral membrane protein (Johnson et al., 2008). The interaction with cytoplasmic proteins renders it difficult to then understand

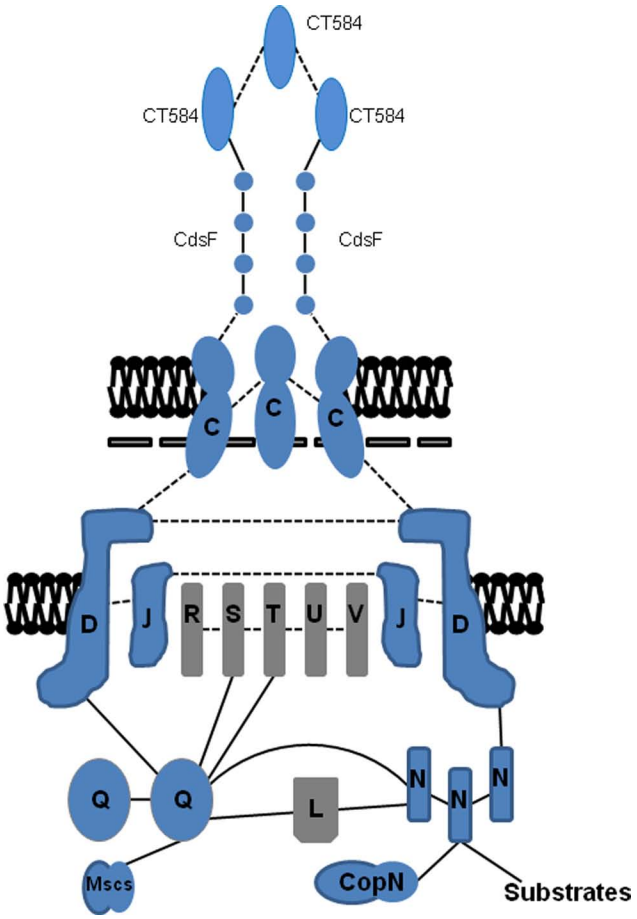

FIGURE 3 | Summary model of protein–protein interactions among chlamydial T3SS components. Respective proteins are placed based on homology to components in other T3SSs (gray) or based on published *in vitro* or *in vivo* data in *Chlamydia* (blue). Interactions are depicted as dashed lines for predicted interactions or solid lines for those that have been established experimentally. Flagellar homologs are omitted for clarity.

how CdsD could interact with CdsC to serve its presumed scaffolding function in the IM ring. Clearly, much more information is required.

INNER MEMBRANE COMPONENTS

Presently, there are only a handful of studies regarding additional components that form the multipartite IM core of the chlamydial NF-T3SS. Homology searches indicate that the composition is similar to the *Yersinia* apparatus with integral IM components consisting of Cds R/S/T/U/V and peripherally IM-associated components Cds L/N/Q (Fields and Hackstadt, 2006). Recent studies relating directly to protein–protein interactions of the chlamydial apparatus components have been particularly illuminating (**Figure 3**). Consistent with other NF-T3SSs, these studies indicated that CdsQ likely forms an important platform for structural integrity and secretion activity of the chlamydial T3SS. CdsQ homologs interact with other IM components of the apparatus to form the inner C-ring complex (Jackson and Plano, 2000; Fadouloglou et al., 2004; Gonzalez-Pedrajo et al., 2006; Morita-Ishihara et al., 2006; Riordan and Schneewind, 2008; Diepold et al., 2010). In *Chlamydia*, association with the IM is apparently mediated by multiple proteins. A combination of GST-fusion co-precipitations and two-hybrid studies using *C. trachomatis* and *C. pneumoniae* proteins were used to identify direct CdsQ interactions with IM components CdsD (Johnson et al., 2008), CdsS, and CdsT (Spaeth et al., 2009). An interaction between CdsQ and CdsV was not detected but instead CdsQ interacted with CT060, the flagella homolog of CdsV (Spaeth et al., 2009). In accordance with its counterparts in *Yersinia* and *Shigella*, *Chlamyida* CdsQ was found to interact with itself (Johnson et al., 2008) and the peripheral IM component CdsL (Johnson et al., 2008; Spaeth et al., 2009). Interestingly, a direct interaction of CdsQ was also detected with CdsN (Stone et al., 2008), a secretion chaperone (discussed below), and currently undefined proteins (Spaeth et al., 2009), further supporting the importance of this protein as an integral linchpin in the chlamydial NF-T3S process.

The close association of CdsL and CdsN warrants further exploration. *Chlamydia* CdsN is homologous to the T3S family of ATPases having similarity to the β subunit of the F_0F_1 ATPases (Hueck, 1998). This class of ATPases plays an important role in release of effectors from their chaperones and unfolding effectors at the IM to render them secretion competent (Akeda and Galan, 2004). *C. pneumoniae* CdsN oligomerizes in solution and hydrolyzes ATP in a time- and dose-dependent manner (Stone et al., 2008). CdsN interaction with CdsL likely facilitates CdsL-mediated regulation of CdsN by inhibiting its ATPase activity (Blaylock et al., 2006; Stone et al., 2008). In addition to interactions with CdsL and CdsQ, potentially novel interactions (Stone et al., 2008) have been detected with apparatus components CdsD, CdsO, and CopN (discussed below). In accordance with putative function in modulating effector folding, a weak interaction between *C. trachomatis* CdsN and the apparent effector protein CT621 (Hobolt-Pederson et al., 2009) was detected in yeast two-hybrid studies (Spaeth et al., 2009). These interactions are likely dynamic *in vivo* and would orchestrate regulated donation of secretion substrates to the T3S apparatus.

Many of the IM components of the virulence-associated T3SS are homologous with IM components of the flagellar T3SS. Intriguingly a recent study has demonstrated interactions between putative flagella homologs and several of the IM components of the *C. pneumoniae* T3SS (Stone et al., 2010). The presence of a handful of flagella genes in chlamydial genomes represents an enigma (Fields and Hackstadt, 2006; Peters et al., 2007). In *C. trachomatis*, CT060 (CdsV paralog) encodes an ortholog of FlhA, an essential integral membrane component of the flagella basal body ring. CT061 is orthologous to FliA, the flagella sigma factor (Macnab, 2004). A second small cluster comprises CT717–CT719 encoding orthologs of FliI (CdsN paralog), the flagella ATPase, a homolog of FliH (CdsL paralog) which is a negative regulator of FliI, and FliF (CdsJ paralog) the MS ring protein, respectively (Macnab, 2004; Peters et al., 2007; Stone et al., 2010). In *C. pneumoniae* several interactions between putative flagella components were observed (Stone et al., 2010). FliI was found to have ATPase activity similar to CdsN, and interacted with the cytoplasmic domain of FlhA. FlhA in turn interacted with the C-terminal region of FliF. Cpn0859 (a FliH homolog by PSI-BLAST analysis) was found to homodimerize and interact with FliI and FlhA in pull-down assays. These findings are all consistent with counterparts from other flagella systems. Although an interaction between the putative FliH homolog Cpn0859 and the ATPase FliI was established, the authors concluded that Cpn0859 is not a FliH ortholog since it was not found to have regulatory function. However, rigorous PSI-BLAST scores argue for the FliH designation and lack of activity could simply be due to technical failure of an *in vitro* system. Certainly the most interesting aspect of this study is derived from interactions identified between the flagella orthologs and IM components of the NF-T3SS. Using the Bacterial-2-hybrid system and co-purification assays, identified interaction partners included FliI-CdsL, FlhA-CdsL, FliI-CopN, FlhA-CdsU (Stone et al., 2010), and FlhA-CdsQ (Spaeth et al., 2009). The fact that *Chlamydia* are non-motile and do not possess a full repertoire of flagella genes is an exciting conundrum (Abdelrahman and Belland, 2005; Peters et al., 2007; Stone et al., 2010), and a minimalist genome demands function for retained genes. We favor a model where a hybrid apparatus is formed incorporating these flagella components into the basal apparatus. Thus, interchanging key apparatus components could result in functionally distinct machinery that correlates with or is dependent on the chlamydial developmental cycle (Stone et al., 2010). This hypothesis is consistent with the observations that "flagellar" genes are consistently transcribed earlier in *C. trachomatis* (Belland et al., 2003) and *C. pneumoniae* (Maurer et al., 2007) development than corresponding paralogs. Clearly, this area represents another exciting facet of the chlamydial T3SS that requires and warrants further study.

THE EXTRA-CELLULAR INJECTISOME

Once a secretion-competent basal apparatus is completed, components can be exported that form exterior structures. Extracellular components of a prototypical T3SS contain a needle, tip, and a pair of translocator proteins (Mueller et al., 2008). Under non-secreting conditions the needle is "capped" by a tip protein that is likely involved in sensing contact with the host cell and subsequently triggers active secretion. Upon activation, translocator proteins are the first to be exported and are required to form a pore in the cytoplasmic membrane consequently allowing direct translocation of the effector substrates (Mota, 2006; Broz et al., 2007). Bioinformatic and biochemical analyses were used to

demonstrate that surface-localized CdsF polymerizes to form the *Chlamydia* needle (Betts et al., 2008). CdsF is highly conserved among chlamydial species and, similar to other NF-T3S needle proteins, is comprised of two predicted helices connected by a loop region. In addition, CdsF interacts with a heterodimeric chaperone (described below) and *C. trachomatis* CT584 (Spaeth et al., 2009). Although the primary sequence of CT584 lacks similarity to other tip proteins, biophysical characterization and structural modeling indicate that CT584 likely encodes the chlamydial tip protein (Markham et al., 2009). This would be consistent with the interaction with CdsF. Importantly, needle and tip proteins from other systems are antigenic and specific antibodies often confer protection from lethal challenge in animal models (Leary et al., 1995; Matson et al., 2005). However, current studies indicate that neither CdsF nor CT584 induce significant antibody responses in *C. trachomatis* infected humans (Wang et al., 2010). While it remains to be tested whether antibodies that do arise have neutralizing potential, it is entirely possible that, given an absolute dependence on T3SS for growth, chlamydial components have evolved to be less antigenic than counterparts in facultative and extra-cellular organisms.

The T3SS apparatus is completed by translocator proteins which presumably oligomerize to form a hetero-oligomeric pore in the host cell cytoplasmic membrane. In the case of *Chlamydia* this would also include the inclusion membrane. *Chlamydia* spp. potentially encode two sets of translocators and this may make sense if functionally distinct T3SSs occur in chlamydiae as discussed above. Gene products CopB and CopB2 are both secreted via a T3S mechanism (Fields et al., 2005; Subtil et al., 2005). In addition, they resemble translocator proteins in predicted structure, and are encoded downstream of genes encoding homologous chaperones (Scc2 and Scc3) and proteins (CopD and CopD2) having characteristics common to additional translocator proteins (Pallen et al., 1997, 2005). CopB would be predicted to act as a translocator with secreted CopD (Ho and Starnbach, 2005) whereas CopB2 would presumably pair with CopD2. Intuitively a second translocator set would be required since CopB is not transcribed until late-cycle (discussed below). Although CopB could likely enable translocation during invasion, it is unclear how it could function as RBs begin to divide. Unfortunately, endogenous CopB2 (unlike CopB) is not detectable in either *C. trachomatis* (Fields et al., 2005) or *C. pneumoniae* (Lugert et al., 2004) inclusion membranes and does not fractionate as an integral membrane protein (Fields, unpublished). If CopB2 has translocator activity, it is certainly atypical. Some translocators in other systems have been shown to have additional effector functions (Hamaguchi et al., 2008; Hamada et al., 2010) and it is also possible that CopB2 has diverged to accomplish this role. This hypothesis would be supported by apparent co-localization of ectopically expressed *C. pneumoniae* CopB2 with host endoplasmic reticulum (Muller et al., 2008).

CHAPERONES

Molecular chaperones play a pivotal role in T3S via their interaction with translocator and effector molecules in the bacterial cytosol. They are involved in prevention of substrate degradation prior to secretion, delivery of the substrate to the apparatus, prevention of substrate aggregation and premature interaction, and transcriptional regulation of T3S genes (Feldman and Cornelis, 2003). In addition it has been postulated that they are required to maintain substrates in a secretion competent state and may also be involved in coordinating secretion to ensure secreted components are released in the correct order (Akeda and Galan, 2005). T3SS chaperones can be separated into several general classes based on the nature of their cognate substrates and structural conservation. For example Class IA chaperone one effector, Class IB chaperone multiple effectors and both adopt similar homo-dimeric structural folds (Parsot et al., 2003). Class II chaperone translocator proteins are structurally distinct, typically containing three tetratricopeptide repeat (TPR) motifs (Pallen et al., 2003). Recently, a third class (Class III) has been recognized that chaperone extra-cellular filament proteins and assume an extended alpha helical structure (Quinaud et al., 2005; Yip et al., 2005; Quinaud et al., 2007; Betts et al., 2008). Studies identifying a number of T3S chaperones have begun to reveal a uniquely complex picture regarding chlamydial T3SS chaperones. Demonstrated or potential *C. trachomatis* T3SS chaperones include: Scc1, Scc2, Scc3, CdsE, CdsG, CT043, CT260, CT274, and CT663 (Fields and Hackstadt, 2006; Spaeth et al., 2009). This is a suspiciously small number of potential chaperones given the number of predicted secretion substrates. However, it is clear from other systems that not all effectors require chaperones (Page and Parsot, 2002; Feldman and Cornelis, 2003) and a picture is emerging in *Chlamydia* that a single chaperone can indeed associate with multiple T3S substrates.

According to similarities in primary sequence, predicted secondary structure, and deduced biochemical parameters with prototypical chaperones, CT043, Scc1, and CT663 would represent chlamydial Class IA chaperones (Fields and Hackstadt, 2006). Currently, no interaction partners for CT043 have been identified. Generally effectors are encoded close to their chaperones, yet CT043 is flanked by genes involved in glycogen metabolism and DNA replication. However, since several of the chlamydial effectors as well as other chaperones (CT274 and CT260) apparently "stand alone" in the genome, this cannot be considered anomalous. On the contrary, the scattering of type-III related ORFs is certainly one of the distinguishing features of the chlamydial T3SS. It is possible that CT043 represents a multi-cargo effector chaperone, and like CesT's involvement with at least seven *E. coli* effectors, may be capable of interacting with a significant number of T3S substrates (Thomas et al., 2005). Analyses of Scc1 and CT663 confound typical classification. Instead of interacting with secretion substrates, yeast two-hybrid data indicate they interact with each other (Spaeth et al., 2009). Hence Scc1 and CT663 likely constitute a heterodimeric chaperone. Although a corresponding secretion substrate awaits identification, CT663 may play an additional role in regulation (discussed below) through an interaction with RNA polymerase (Rao et al., 2009).

An extensive yeast two-hybrid screen identified a unique chlamydial protein that represents a new Class IB T3S chaperone capable of binding several effectors (Spaeth et al., 2009). CT260/multiple cargo secretion chaperone (Mcsc) is at the center of a protein–protein interaction hub involving three inclusion membrane proteins, Cap1, CT618, and CT225, respectively and the cytoplasmic C-ring of the apparatus, CdsQ. The data suggest that Mcsc is required to stabilize Cap1 and CT618 in the bacterial cytosol and may facilitate their delivery to the ATPase of the secretion appara-

tus via a docking interaction with CdsQ (Spaeth et al., 2009). The Mcsc-effector-CdsQ interaction is not unique to *Chlamydia* since other CdsQ homologs have been shown to be capable of recognizing chaperone-effector complexes (Gonzalez-Pedrajo et al., 2006; Morita-Ishihara et al., 2006; Spaeth et al., 2009). What is unique is that Mcsc alone was able to bind to CdsQ, raising the interesting possibility that Mcsc may be pre-docked at the T3S apparatus. Indeed, Mcsc partitioned within the EB IM in the absence of any effector cargo, and Cap1 and CT618 are synthesized at different time points post-invasion (Spaeth et al., 2009). Speath et al. speculate that either Mcsc dimers detach from CdsQ to stabilize newly synthesized Cap1 and CT618, or more intriguingly, that the transcribing ribosome itself is recruited to the C-ring. If the latter proved to be the case it would be the first time that translational machinery has been shown to directly associate with the T3S apparatus.

Potential chlamydial Class II chaperones include CT274, Scc2, and Scc3 (Fields and Hackstadt, 2006). Despite conclusive sequence similarity with *Yersinia* SycD and the presence of TPR domains, *in silico* analyses reveal some distinguishing features for these proteins. It has previously been noted that T3SS chaperones are often small with an acidic pI (Feldman and Cornelis, 2003). Like homologs in other systems, CT274 is small (15.5 kDa) and has an acidic pI of 4.45, yet Scc2 and Scc3 do not fit either of these criteria at 26 and 23 kDa with pIs of 9.58 and 6.71, respectively. The larger mass corresponds to unique N-terminal regions of approximately 60 residues that share little sequence similarity to each other or other chaperones. Despite these differences, *in vitro* studies demonstrated that both Scc2 and Scc3 were capable of interacting with the *Yersinia* SycD substrate YopD, supportive of a conserved function. Work indicates an interaction of Scc2 with putative translocator proteins CopB (Fields et al., 2005; Spaeth et al., 2009) and CopD (Spaeth et al., 2009). Scc3 has been found to interact with CopB2 (Fields et al., 2005) and with the C-terminal domain of CopN from *C. trachomatis*, *C. pneumoniae* and *C. pscittaci*, respectively (Slepenkin et al., 2005). Similar to Scc2 and Scc3, PSI-BLAST analysis indicates a characteristic TPR domain within CT274. In contrast, specific homology with known T3SS chaperones is not apparent. Work by Spaeth et al. (2009) has identified an interaction of CT274 with CT668 and CT161. CT161 represents a *Chlamydia*-specific hypothetical ORF, whereas the genomic positioning of CT688 would suggest a role in the T3S apparatus, perhaps corresponding to *Yersinia* YscH/YopR (Blaylock et al., 2010). In support of this prediction and the need for a chaperone, the gene product corresponding to CT668 in *C. pneumoniae* is secreted (Subtil et al., 2005; Muller et al., 2008).

Finally, chlamydial T3SS chaperones also include the recently identified Class III needle subunit co-chaperones CdsE and CdsG (Betts et al., 2008). CdsE and CdsG form a heteromeric complex (Betts et al., 2008; Spaeth et al., 2009) required for CdsF stability. Secondary structure analysis of CdsE and CdsG demonstrated conserved features with other needle chaperones and gene order was conserved with *Yersinia*, *Pseudomonas*, and *Bordetella* (Betts et al., 2008). This is thus another example of chlamydial sequence divergence with functional and "genetic neighborhood" conservation (Betts et al., 2008; Lorenzini et al., 2010). This theme is further exemplified by the recently crystallized CT670, a *Chlamydia*-specific protein with no identifiable homologs outside of *Chlamydia* spp. (Gupta and Griffiths, 2006; Lorenzini et al., 2010). Located in the same T3SS gene cluster as CdsD/E/F/G, gene order conservation suggests CT670 may be analogous to YscO, an essential multifunctional component of the *Yersinia* T3SS that is required for effector export and secretion of the "molecular ruler" protein YscP (Journet et al., 2003; Riordan et al., 2008). Lorenzini et al. provided compelling *in silico* and *in vitro* evidence, including protein crystallization, that CT670 is indeed a YscO homolog and should now be considered CdsO. In addition, a chaperone function for CdsO is supported by the demonstration of CdsO interaction with the secreted protein CT671 (Subtil et al., 2005) which likely represents the homolog of *Yersinia* YscP. CdsO was also found to interact with itself in *C. pneumoniae* (Stone et al., 2008); thus being consistent with other T3S chaperones which typically dimerize (Page and Parsot, 2002). Interestingly, an interaction between CdsO and the ATPase CdsN was demonstrated in *C. pneumoniae*. This lead to speculation that *C. pneumoniae* CdsO may also act as a chaperone for CdsN (Stone et al., 2008). However, since there is considerable evidence that *Yersinia* YscO is also capable of interactions with IM T3S components (Riordan and Schneewind, 2008) and co-purified with a complex containing the *Yersinia* ATPase YscN (Riordan et al., 2008), it is possible that this interaction represents additional functional roles for CdsO distinct from chaperone activity.

REGULATION OF T3SS

REGULATION OF GENE EXPRESSION

Chlamydial gene transcription is temporally regulated by incompletely defined mechanisms and is generally classified as early-, mid-, and late-cycle. These times correspond roughly to key developmental events such as EB to RB conversion, active RB replication, and differentiation of RBs back to EBs, respectively (Abdelrahman and Belland, 2005). An apparent paradox in the early days of chlamydial T3S was the fact that *de novo* synthesis of apparatus components generally does not begin until mid-cycle. Moreover, genes encoding Scc2, CopB, and CopD are not transcribed until late-cycle (Fields and Hackstadt, 2006). Considerable work, including identification of EB-localized T3S proteins (Vandahl et al., 2001; Fields et al., 2003) and secretion of T3S effectors such as TARP (Clifton et al., 2004) and CT694 (Hower et al., 2009) during invasion, indicate that a T3SS is present and functional throughout chlamydial development. A reasonable model predicts that preformed T3SSs in EBs mediate effector secretion during invasion and early development until replaced by *de novo* synthesized systems as RBs begin to divide.

Clues are beginning to emerge regarding how modulation of T3S gene expression is accomplished. RT-PCR transcriptional linkage analysis profiling of six disseminated T3SS-encoding loci revealed seven polycistronic operons preceded by predicted *E. coli* σ^{70}-like promoter elements (Hefty and Stephens, 2007). Interestingly, quantitative gene expression provided evidence for three internal operons also possessing putative σ^{70}-like promoters. These internal operons would result in expression of (i) *cdsEFGHNOPQ*, (ii) *cdsJLRST*, and (iii) *cdsC* independent from CT663-*cdsD*, *lpdA-lipA*, and *pkn5*, respectively. It is currently unclear what would necessitate this separation. Transcriptional regulation of T3S genes in other organisms is primarily controlled by AraC-like transcriptional activators which act in response to environ-

mental cues encountered during infection; orthologs of which are conspicuously absent from the chlamydial genome (Darwin and Miller, 1999; Rakeman et al., 1999; Mavris et al., 2002; Akbar et al., 2003; Case et al., 2010). Recent data suggests that there are no explicit transcriptional activators of chlamydial T3S operons and that their temporal expression is under the control of general mechanisms. Specifically it was found that, as predicted by previous studies (Hefty and Stephens, 2007), promoters for all ten of the T3SS operons were transcribed *in vitro* by σ^{66}, the major form of chlamydial RNA polymerase. Furthermore, these promoters were differentially sensitive to alterations in DNA supercoiling that corresponded to *in vivo* temporal expression patterns (Di Russo Case et al., 2010). Transcription of T3S mid-cycle promoters was found to be activated in response to an increase in DNA supercoiling in a similar manner to other chlamydial mid-cycle promoters. Conversely, late cycle T3S promoters were insensitive to DNA supercoiling in accordance with several other supercoiling-independent, σ^{66}-regulated chlamydial late genes (Case et al., 2010). This apparent lack of T3SS-specific transcriptional activators certainly makes intuitive sense for the coordination of T3SS expression with the overall *Chlamydia* developmental cycle.

REGULATION OF SECRETION ACTIVITY

The chlamydial T3SS is activated within minutes of contact with a eukaryotic cell (Clifton et al., 2004) and likely remains active so long as chlamydiae remain attached to the inclusion membrane (Fields and Hackstadt, 2006). Similar to other T3SSs (Hueck, 1998), the chlamydial system can therefore correctly be referred to as contact-dependent. But what factors directly govern secretion activity? Several clues now exist, and initial attachment to the host plasma membrane likely represents the activation signal. In direct support of this notion, an *in vitro* release assay was adapted that was capable of stimulating release, albeit inefficiently, of invasion-related Tarp from cell-free EBs (Jamison and Hackstadt, 2008; Spaeth et al., 2009). Importantly, the stimuli, including the Ca^{2+} chelator EGTA, are also functional for other contact-activated T3SSs (Kim et al., 2005). It should be noted that activation of the chlamydial T3SS is likely more complicated given the metabolic inactivity and disulfide-bonded envelope of EBs. For example, we have emphasized that the presence of cysteine residues in CdsF is a feature unique to the chlamydial T3SS (Betts et al., 2008). Indeed, we find that EB, but not RB, CdsF cysteines are disulfide bonded (Betts-Hampikian, unpublished). If these bonds interfere with secretion, reduction of EB-localized bonds would likely be required before full activity could be achieved.

Activation of secretion would also likely require concomitant secretion of CopN. Although secretion to the inclusion membrane is evident later in development (Fields and Hackstadt, 2000), complications in immunolocalization sensitivity currently confound efforts to demonstrate early export. CopN contains domains similar to *Yersinia* YopN and TyeA, two proteins involved in negative regulation of T3S. According to one plausible *Yersinia* model (Joseph and Plano, 2007), YopN is directed to the injectisome by the heterodimeric chaperone SycN/YscB but its secretion is prevented by interaction with the apparatus through TyeA. Once the appropriate stimuli are detected by the needle/tip complex, the interaction with TyeA is dissolved, YopN is secreted, and the apparatus is competent for secretion. The interaction of Scc3 with CopN noted above occurs through the domain having similarity to TyeA. Therefore, this could mediate CopN mobilization to the T3S apparatus or other, yet to be identified chaperones similar to SycN/YscB, could be involved. It is formally possible that the interaction of CopN with CdsN could also play a role (Stone et al., 2008), but we favor an interpretation that this interaction merely reflects CdsN association with secretion substrates. Once CopN is secreted, it is unclear if substrate switching would then occur in *Chlamydia* spp. Although chlamydial genomes contain CdsU, it has not been determined whether this protein is cleaved similarly to *Yersinia* YscU to allow secretion of effector proteins (Riordan and Schneewind, 2008).

Secretion of mid- and late-cycle effectors (Valdivia, 2008; Betts et al., 2009) indicate secretion activity that is sustained presumably until an RB dissociates from the inclusion membrane during differentiation to an EB. The environmental cues that result in this event remain undefined, but Wilson et al. (2009) have suggested that something as simple as space constraints within the inclusion dictate dissociation. Electron micrographs from multiple groups (Matsumoto, 1982a; Nichols et al., 1985) contain images of cylindrical surface projections emanating from RBs and forming direct connection with the inclusion membrane. Presuming that these structures correspond to CdsF-containing T3S filaments, enumeration studies suggest that intra-inclusion space constraints result in fewer T3SSs making contact with a eukaryotic membrane (Wilson et al., 2006). This possibility raises a multitude of questions. For example, does CT671 correspond to a molecular ruler similar to *Yersinia* YscP (Journet et al., 2003; Riordan et al., 2008) and could it be involved in retracting CdsF-containing needles? Alternatively, is a more direct physical dissociation responsible for loss of contact? Since CT584 is a late-cycle gene product, does its *de novo* production result in capping of needle filaments to cease secretion activity? Is secretion activity responding to dissociation or causing it? Are the uniquely late-cycle Scc2, CopB, and CopD involved in the process? Regardless of the precise mechanism, T3SS activity clearly corresponds to chlamydial development and represents an active area of current investigation.

ADDITIONAL ROLES IN PATHOGENESIS

As already noted, one way the T3S apparatus contributes directly to chlamydial pathogenesis is through secretion of effector proteins affecting host cellular processes (Valdivia, 2008; Betts et al., 2009) and immunostimulatory capability (Prantner and Nagarajan, 2009). The secretion system may contribute indirectly to the host immune response, and therefore to immunopathology, since injectisome components such as CopN elicit significant antibody responses during natural infection (Wang et al., 2010). It is additionally postulated, and highly likely, that the chlamydial T3SS is intimately involved in mediation of intracellular development and absolutely essential to the developmental cycle; a concept that is in contrast to other T3SS containing organisms whereby the secretion system is essential for virulence but dispensable for survival (Wilson et al., 2006; Peters et al., 2007). We acknowledge that separation of direct apparatus-mediated affects from developmental affects resulting from downstream responses to activities of secreted effectors will be complex. The impact(s) of

secretion and substrate activities are likely intimately entwined in the chlamydial system. Possibilities for direct roles of the apparatus do, however, exist. First, it is possible that the T3S apparatus contributes to maintaining attachment of chlamydiae to eukaryotic membranes. We feel that it is unlikely that the T3SS is the sole mediator of initial attachment to plasma membranes. This concept is rare for T3SSs (Lara-Tejero and Galan, 2009) and evidence for a plethora of chlamydial attachment factors is abundant in the literature (Campbell and Kuo, 2006). Moreover, distinct variations exist among attachment requirements of different *Chlamydia* serovars and species (Dautry-Varsat et al., 2005) while surface-exposed T3S components are highly conserved. However, it is much more likely that the T3S apparatus is directly involved in stable association with the inclusion membrane. Structures likely corresponding to T3S needles (Matsumoto, 1982a; Nichols et al., 1985) conspicuously protrude through the inclusion membrane to potentially tether RBs. As noted above, these observations led to the hypothesis that T3S activity directly controls differentiation of RBs back into infectious EBs (Peters et al., 2007). In addition to the discussed mathematical models (Wilson et al., 2006), there is direct evidence to suggest that this may be the case. The recent discovery that certain acylated hydrazones of salicylaldehydes represent specific, small molecule inhibitors of T3SSs (Keyser et al., 2008) has provided a potentially valuable tool to investigate contributions of T3S to chlamydial development. We (Wolf et al., 2006) and others (Muschiol et al., 2006; Bailey et al., 2007; Slepenkin et al., 2007) have shown that treatment of *Chlamydia* infected cultures with these inhibitors interferes with T3S and inhibits productive development of chlamydiae. It is not currently possible to rule out potential T3SS-independent effects on *Chlamydia* spp., and indirect effects such as chelation of iron likely contribute to the ability of inhibitors to interfere with chlamydial growth (Slepenkin et al. 2007). However, these studies are consistent in that drug treatment prevents conversion of RBs to EBs, reinforcing the possibility for a direct link between the T3SS and development. In addition, independent observations that (i) ectopic expression of the T3S effector IncA blocks development of *C. caviae* (Alzhanov et al., 2004), (ii) microinjection of neutralizing antibodies specific for the T3S effector CT229 arrest *C. trachomatis* development (Hackstadt et al., 1999; Rzomp et al., 2006), and (iii) inhibition of CopN interferes with *C. pneumoniae* growth (Huang et al., 2008) are all consistent with a predominant and direct role of T3S in developmental progression. Finally, findings that the putative Class I chaperone CT663 can exert a negative regulatory effect on σ^{66}-dependent transcription (Rao et al., 2009) provide potential mechanisms by which secretion activity could feed-back to govern chlamydial gene expression on a global scale.

CONCLUDING REMARKS

One may reasonably question the logic and sanity of pursuing characterization of a complex secretion system in a genetically intractable organism. We have endeavored herein to provide an overview of current data describing the chlamydial T3SS and make forward-looking interpretations regarding unique biology. We certainly acknowledge that some of the discussed work represents preliminary characterization and the overall picture is far from complete. Indeed some studies, particularly those involving interaction studies of ectopically expressed proteins, should be viewed with a level of caution. Given the unique physiology involved it is probably not possible to reconstitute the chlamydial T3SS in an amenable host as was done for the *Yersinia* system (Bartra et al., 2006). As the field continues to mature however, the weight of accumulated evidence will doubtless validate findings that are physiologically relevant. Clearly, qualities regarding the chlamydial T3S apparatus remain to be elucidated. For example, several components of the basal apparatus are conspicuously absent from the chlamydial T3SS. A homolog of YscK and the inner rod protein YscI are not readily apparent. Whether these absences represent proteins that are present but remain to be discovered or are further examples of divergence of the chlamydial system remains to be seen. However, the most interesting studies will likely reveal how this intricate system directly responds to and/or controls chlamydial development and therefore overall virulence.

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Chlamydia pneumoniae CdsL regulates CdsN ATPase activity, and disruption with a peptide mimetic prevents bacterial invasion

Chris B. Stone[1], David C. Bulir[1], Connor A. Emdin[1], Ryan M. Pirie[1], Elisa A. Porfilio[1], Jerry W. Slootstra[2] and James B. Mahony[1]*

[1] Michael G. DeGroote Institute for Infectious Disease Research, Faculty of Health Sciences, Department of Pathology and Molecular Medicine, McMaster University, Father Sean O'Sullivan Research Centre, St. Joseph's Healthcare, Hamilton, ON, Canada
[2] PepScan Presto, Epitope Mapping, Lelystad, Netherlands

Edited by:
Rey Carabeo, Imperial College London, UK

Reviewed by:
Agathe Subtil, Pasteur Institute, France
Samantha Gruenheid, McGill University, Canada

***Correspondence:**
James B. Mahony, Regional Virology and Chlamydiology Laboratory, St. Joseph's Hospital, L305, 50 Charlton Avenue East, Hamilton, ON, Canada L8N 4A6.
e-mail: mahonyj@mcmaster.ca

Chlamydiae are obligate intracellular pathogens that likely require type III secretion (T3S) to invade cells and replicate intracellularly within a cytoplasmic vacuole called an inclusion body. *Chlamydia pneumoniae* possess a YscL ortholog, CdsL, that has been shown to interact with the T3S ATPase (CdsN). In this report we demonstrate that CdsL down-regulates CdsN enzymatic activity in a dose-dependent manner. Using Pepscan epitope mapping we identified two separate binding domains to which CdsL binds viz. $CdsN_{221–229}$ and $CdsN_{265–270}$. We confirmed the binding domains using a pull-down assay and showed that GST–$CdsN_{221–270}$, which encompasses these peptides, co-purified with His–CdsL. Next, we used orthology modeling based on the crystal structure of a T3S ATPase ortholog from *Escherichia coli*, EscN, to map the binding domains on the predicted 3D structure of CdsN. The CdsL binding domains mapped to the catalytic domain of the ATPase, one in the central channel of the ATPase hexamer and one on the outer face. Since peptide mimetics have been used to disrupt essential protein interactions of the chlamydial T3S system and inhibit T3S-mediated invasion of HeLa cells, we hypothesized that if CdsL–CdsN binding is essential for regulating T3S then a CdsN peptide mimetic could be used to potentially block T3S and chlamydial invasion. Treatment of elementary body with a CdsN peptide mimetic inhibited *C. pneumoniae* invasion into HeLa cells in a dose-dependent fashion. This report represents the first use of Pepscan technology to identify binding domains for specific T3S proteins viz. CdsL on the ATPase, CdsN, and demonstrates that peptide mimetics can be used as anti-virulence factors to block bacterial invasion.

Keywords: type III secretion, peptide mimetic, *Chlamydia*, ATPase, Pepscan

INTRODUCTION

Chlamydia pneumoniae is an obligate, intracellular Gram-negative bacteria that has been associated with community acquired pneumonia (Clifton et al., 2004), atherosclerosis (Grayston, 2000), arthritis (Ardeniz et al., 2005), and Alzheimer's disease (Balin et al., 2008). The members of the genus *Chlamydia* all share a unique, biphasic life-cycle that is initiated by attachment of the metabolically quiescent elementary body (EB) to the host cell. The association between the EB and the host cell membrane is poorly understood, but glycosaminoglycans may be involved (Chen and Stephens, 1997). Once attached to the host cell, type III secretion (T3S) is utilized to inject the translocated actin recruitment protein (TARP) to facilitate bacterial internalization into a plasma-membrane derived vacuole, termed an inclusion (Clifton et al., 2004; Lane et al., 2008). The MEK–ERK and PI 3-kinase pathways of the host cell are also involved in bacterial uptake, and are possibly targets of other T3S effectors translocated across the cytoplasmic membrane upon EB contact and binding to host cells (Coombes and Mahony, 2002; Carabeo et al., 2004; Subtil et al., 2004). Once inside the host cell the remainder of the life-cycle occurs inside the inclusion body where EBs differentiate into the metabolically active, non-infectious reticulate bodies (RB). The RB remains in close association with the inclusion membrane, suggesting a need for active T3S in RBs. This interaction with the inclusion membrane allows the RB to communicate with the host cell via T3S, allowing the *Chlamydia* to commandeer host cell pathways to obtain lipids, cholesterol, and other nutrients crucial for its growth and replication, and also to prevent phagosome endosome fusion (Wylie et al., 1997; Hoare et al., 2008; Scidmore and Hackstadt, 2008). Following inclusion body growth to accommodate replicating RBs, an unknown signal (possibly quorum sensing) triggers the detachment of RBs from the membrane and subsequent re-assortment into infectious EBs. The EBs then exit the host cell either by cell lysis or through extrusion, a packaged release mechanism which leaves the host cell intact (Hybiske and Stephens, 2007).

Type III secretion is a virulence mechanism commonly used by Gram-negative bacteria to directly translocate effector proteins from the bacterial cytoplasm to the host cell cytoplasm in a single step, through the use of a syringe-like apparatus termed an injectisome (Galan and Collmer, 1999; Ghosh, 2004; Galan and Wolf-Watz, 2006). The injectisome is constructed of 20–25 proteins spanning the inner membrane, periplasm, and outer membrane, extending into the extracellular milieu to allow for host cell sensing and contact. Upon host cell contact, the T3S injectisome apparatus

injects two translocator proteins into the host cell membrane to form the translocon, a molecular pore through which secreted proteins can enter the host cell (Goure et al., 2004). Despite the fact that *Chlamydia* possesses a full repertoire of T3S genes scattered throughout the genome on at least 10 distinct operons, a systematic study of the injectisome has yet to be undertaken as chlamydial species are genetically intractable (Hefty and Stephens, 2007). Recent reports have identified a few T3S structural and effector proteins, providing some understanding of the *Chlamydia* T3SS. Our laboratory has shown that CdsD, a unique protein orthologs to YscD that contains two fork-head associated domains, interacts with the predicted *C. pneumoniae* ATPase tethering protein, CdsL, as well as CdsQ, a putative multi-cargo transport protein (Johnson et al., 2008; Spaeth et al., 2009). We extended these findings to show that CdsN also binds to this complex, and interacts independently with CdsD, CdsL, and CdsQ (Stone et al., 2008). Betts et al. (2008) have also recently identified the *C. trachomatis CT666* gene encoding CdsF, the needle filament protein of the injectisome. The role of a few effector proteins of *C. pneumoniae* have been recently discovered. Cpn0585 is found in the inclusion membrane and interacts with Rab 1, 10, and 11, possibly recruiting these ATPases to the inclusion membrane (Cortes et al., 2007). Cpn0827 is a second effector protein that is detectable in the inclusion membrane at 20 hpi (Hermann et al., 2006). The *Chlamydia* protein associating with death domains (CADD) is a conserved effector known to interact with TNF family receptors (Stenner-Liewen et al., 2002). Recent biophysical evidence has also been presented by Markham et al. (2009) suggesting that the *C. trachomatis* protein CT584 could function as the needle tip protein, which is imperative for host cell sensing and translocator insertion into the host cell membrane.

Type III secretion ATPases are believed to be assembled as a hexameric ring at the basal body of the injectisome and play a role in delivery of effector proteins through the injectisome (Zarivach et al., 2007). Several T3S ATPases have been partially characterized, including EscN from *Escherichia coli* (Andrade et al., 2007), YscN from *Yersinia* (Blaylock et al., 2006), and InvC from *Salmonella* (Akeda and Galan, 2004), although the ability of the T3S ATPase to coordinate numerous protein interactions has not been explored. These ATPases have been shown to have significant sequence orthology to the β subunit of the F_0F_1 ATPase, and to hydrolyze ATP. Not only are these ATPases important for providing energy for protein transport, but they are believed to play a role in unfolding the effector proteins before translocation, which may be accomplished by releasing the chaperone from its cognate effector protein (Akeda and Galan, 2005). In *Yersinia*, the ATPase tethering protein YscL localizes the ATPase to the inner membrane, potentially by performing a function similar to that of the gamma-stalk of the F_0F_1 ATPase (Pallen et al., 2006). YscL has also been shown to play a role in regulation of the ATPase activity by down-regulating enzymatic activity (Blaylock et al., 2006). The quaternary structure of ATPase, viz. the hexamer, has been associated with enhanced enzymatic activity and most likely reflects the native conformation of this protein.

In this report we show that CdsL down-regulates the enzymatic activity of CdsN. We utilized Pepscan epitope mapping to determine where CdsL binds to CdsN, and used an orthology modeling approach to map this domain onto the predicted 3D structure of CdsN (Bernard et al., 2004; Teeling et al., 2006; Timmerman et al., 2007). We found that CdsN possesses two distinct binding domains for CdsL, one to potentially mediate the tethering function and the other to mediate the down-regulation function. We also show that a CdsN peptide acts as a peptide mimetic preventing bacterial invasion. This is the first report to identify binding domains for a T3S ATPase regulating protein and the use of a peptide mimetic as a novel antimicrobial agent to potentially target T3S.

MATERIALS AND METHODS

EXPRESSION PLASMIDS

Chlamydia pneumoniae CWL029 (VR1310:ATCC; GenBank accession No. AE001363) was the strain used to isolate genomic DNA for cloning and protein expression. Full length *cdsL* and *cdsN* were amplified from CWL029 using AttB-containing primers (Gateway; Invitrogen). The amplified products were cloned into $pDONR_{201}$ (Gateway; Invitrogen) to generate pENT vectors. The pENT vectors were then used in LR reactions (Gateway; Invitrogen) to produce pEX vectors containing the genes of interest. We used either pEX_{17} (N-terminal His-tag) or pEX_{15} (N-terminal GST-tag) vectors for our protein expression. All constructs were confirmed by sequencing at the Molecular Biology Facility at McMaster University.

PROTEIN EXPRESSION

All constructs were expressed in *E. coli* Rosetta pLysS. Expression plasmids were used to transform *E. coli* Rosetta pLysS and plated on LB plates containing 100 μg/mL ampicillin. LB broth (750 mL), containing antibiotics, was then inoculated with 12 mL of an overnight culture and grown at 37°C until they reached an optical density $(OD)_{600}$ of approximately 0.8. Cultures were then cooled on ice to 20°C and induced with 0.2 mM of isopropyl β-D-thiogalactopyranoside (IPTG). Cultures were then incubated at 23°C for 2 h and bacteria were harvested by centrifugation at 6500×*g* for 10 min in a Sorvall RC-5B centrifuge and washed with ice-cold phosphate-buffered saline (PBS). Bacteria containing His-tagged protein were resuspended in either Binding Buffer (50 mM potassium phosphate pH 7.2, 150 mM KCl, 1 mM $MgCl_2$) when used in GST pull-downs, or Nickel A buffer (20 mM Tris pH 7.0, 0.02% β-mercaptoethanol, 400 mM KCl, 1% Triton X-100) when used in ATPase activity assays, while the bacteria containing GST-tagged protein were resuspended in PBS and stored at −20°C until further use.

PURIFICATION OF RECOMBINANT PROTEINS

Escherichia coli pellets containing over-expressed His- or GST-tagged proteins were thawed on ice and sonicated using a Fischer Scientific Sonic Dismembrator Model 100, followed by centrifugation at 20,000×*g* for 40 min to remove insoluble material. Supernatants containing His-tagged protein for use in GST pull-down assays were stored at 4°C. GST-tagged protein for use in GST pull-down assays were bound to 300 μL of glutathione beads overnight at 4°C, then blocked overnight in Tris Buffered Saline with 0.1% Tween-20 and 4% BSA and stored at 4°C until use. His- and GST-tagged protein supernatants for use in ATPase activity assays were filtered through 0.45 μm acrodisc filters (Pall Corporation) and purified on either a 1-mL GSTrap FF column (GE Healthcare) or a 1-mL HisTrap HP column (GE Healthcare). For GST-tagged protein, columns

were washed with PBS + 0.1% Tween until the flow-through had an OD_{280} of less than 0.005. GST-tagged protein was then eluted off the beads using 1.5 μg/μL reduced glutathione (Sigma) and dialyzed against activity buffer (50 mM Tris–HCl pH 7.0, 5 mM $MgCl_2$, 10 mM KCl). Purity was confirmed using SDS–PAGE and Coomassie blue staining. For His-tagged proteins, columns were washed with increasing imidazole concentrations and eluted with 300 mM imidazole then dialyzed into activity buffer. Purity was confirmed using SDS–PAGE and Coomassie blue staining.

CdsN ENZYMATIC ACTIVITY DOWN-REGULATION EXPERIMENTS

ATP hydrolysis by GST–CdsN purified from GSTrap columns was measured using a malachite green assay (R&D Systems) which measures released inorganic phosphate from ATP. For all experiments, the specific activity was determined using the equation of a standard line generated using phosphate standard (R&D Systems). Reaction mixtures contained 150 ng of GST–CdsN, 40 μM ATP, 50 mM Tris–HCl pH 7.0, 5 mM $MgCl_2$, and 10 mM KCl. The reaction mixture (1 mL) was incubated at 37°C for 1 h and 50 μL of the mixture was taken for inorganic phosphate determination at various time points. The reaction was stopped by the addition of 10 μL of Malachite Green Reagent A followed by 10 μL of Malachite Green Reagent B and incubated at room temperature for 1 min before an OD_{610} reading was taken, according to the manufacturer's instructions. For the negative control, purified CdsN was digested for 10 min at 37°C using Proteinase K (Invitrogen). Also, as a negative control, another GST-tagged protein (CopN) that lacks ATPase activity was purified in the same manner and tested for activity. ATPase activity was expressed as nmol phosphate released min^{-1}, and all experiments were performed in triplicate. His–CdsL was purified from Ni-NTA agarose beads and tested for any contaminating ATPase activity using the Malachite Green Assay. It was then added in varying amounts into the reaction mixture with CdsN prior to addition of ATP and incubated for 20 min on ice. The activity assay was then performed as described above.

GST PULL-DOWN ASSAYS

To examine the interaction of $CdsN_{221–270}$ with CdsL, GST pull-down assays were performed as described previously by Johnson et al. (2008), with the following modifications. Briefly, glutathione–agarose beads (30 μL) bound to 50 ng of GST-tagged $CdsN_{221–270}$ protein was used in the assay. The beads were incubated overnight at 4°C with the *E. coli* lysate expressing the His-tagged proteins. The beads were collected by centrifugation and washed with 0.1% Triton X-100 and increasing concentrations of NaCl to eliminate spurious protein interactions. The presence of the GST- and His-tagged proteins were confirmed by both Coomassie blue and Western blot. All proteins were eluted from the glutathione beads and electrophoresed on an 11% SDS–PAGE gel before being probed for His-tagged protein. GST alone bound to glutathione beads was used as a negative control for the pull-down.

PEPTIDE LIBRARY SYNTHESIS, SCREENING ASSAYS, AND 3D MODELING

The linear and/or CLIPS peptides are synthesized based on the amino acid sequence of the target protein using standard Fmoc-chemistry and de-protected using trifluoric acid with scavengers. The constrained peptides are synthesized on chemical scaffolds in order to reconstruct conformational epitopes, using Chemically Linked Peptides on Scaffolds (CLIPS) technology (Timmerman et al., 2007). For example, the single looped peptides are synthesized containing a dicysteine, which was cyclized by treating with alpha, α′-dibromoxylene, and the size of the loop is varied by introducing cysteine residues at variable spacing. If other cysteines besides the newly introduced cysteines are present, they are replaced by alanine. The side-chains of the multiple cysteines in the peptides are coupled to CLIPS templates by reacting onto credit-card format polypropylene Pepscan cards (455 peptide formats/card) with a 0.5-mM solution of CLIPS template such as 1,3-bis (bromomethyl) benzene in ammonium bicarbonate (20 mM, pH 7.9)/acetonitrile [1:1(v/v)]. The cards are gently shaken in the solution for 30–60 min while completely covered in solution. Finally, the cards are washed extensively with excess of H_2O and sonicated in disrupt-buffer containing 1% SDS/0.1% β-mercaptoethanol in PBS (pH 7.2) at 70°C for 30 min, followed by sonication in H_2O for another 45 min. The binding of antibody or labeled protein/peptide to each peptide is tested in a Pepscan-based ELISA. The 455-well credit-card format polypropylene cards containing the covalently linked peptides are incubated with peptide solution for example consisting of 1 μg/mL diluted in blocking solution, for example 4% horse serum, 5% ovalbumin (w/v) in PBS/1% Tween. After washing, the peptides are incubated with a monoclonal mouse anti-His-tag antibody (1/1000, Novagen, 70796-3) and subsequently after washing with a rabbit-anti-mouse antibody peroxidase conjugate (1/1000, Southern Biotech, 6175-05) for 1 h at 25°C. After washing, the peroxidase substrate 2,2′-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) and 2 μL of 3% H_2O_2 are added. After 1 h, the color development is measured. The color development is quantified with a charge coupled device (CCD)-camera and an image processing system. The raw-data are optical values obtained by a CCD-camera. The values mostly range from 0 to 3000, a log scale similar to 1–3 of a standard 96-well plate ELISA-reader. First the CCD-camera makes a picture of the card before peroxidase coloring and then again a picture after the peroxidase coloring. These two pictures are subtracted from each other which results in the data which is called raw-data. This is copied into the Peplab™ database. Then the values are copied to excel and this file is labeled as raw-data file. One follow-up manipulation is allowed. Sometimes a well contains an air-bubble resulting in a false-positive value, the cards are manually inspected, and any values caused by an air-bubble are scored as 0. 3D structure prediction was performed using 3D-JIGSAW (http://www.bmm.icnet.uk/~3djigsaw/). Modeling was then performed using PYMOL software.

PEPTIDE DESIGN AND ENTRY INTO EBs

Based on the Pepscan epitope mapping of CdsN, two domains were found that mediate the interaction between CdsN and CdsL. The smaller of the two domains (residues 265–279), corresponding to TRFARA, was chosen to create a peptide mimetic compound. We synthesized the TRFARA peptide with a N-terminal membrane transport sequence (MTS), YGRKKRRQRRR (Efthymiadis et al., 1998). We also extended the CdsN sequence flanking the TRFARA peptide to ensure the entire binding domain was included, and flanked the peptide with cysteine residues to assist with any disulfide bonding that may be required for binding.

The final peptide utilized was the 28-amino acid sequence YGRKKRRQRRRCVVLMMDSVTRFARALC and was used in its linear form for inhibition experiments. Peptides were synthesized by Peptide 2.0.

GST–MTS UPTAKE BY EBs

Chlamydial EBs were purified using a discontinuous gastrografin gradient, and resuspended in PBS. Either GST or GST–MTS was incubated at varying concentrations (0, 100, 500 μm) or for varying times (5, 30, or 60 min) with the purified EBs at 4°C. The EBs were then pelleted and washed three times with PBS, followed by trypsinization for 30 min to ensure that no protein was bound to the outside of the EB. EBs were then boiled in loading dye and probed for the presence of GST using Western blot.

PEPTIDE CYTOTOXICITY

The cytotoxicity was performed as described by Johnson et al. (2009). The effect of the CdsN TRFARA peptide on HeLa cell viability was determined. Briefly, 50 or 100 μM of the CdsN peptide, control peptides, or the positive control (1% SDS plus 0.1% Triton X-100) were added to sub-confluent HeLa cells in six-well plates. At 1 h supernatants were harvested and tested for the presence of adenylate kinase using a cytotoxicity assay (Lonza ToxiLight® BioAssay, Rockland). The cytotoxicity assay was performed as per the manufacturer's protocol. Briefly, supernatants from HeLa cell cultures incubated with the CdsN TRFARA peptide (in MEM containing cycloheximide) were tested for evidence of eukaryotic cell cytotoxicity. Aliquots (5 μL) of each supernatant were mixed with 25 μL of Adenylate Kinase Detection Reagent and samples were incubated at room temperature for 5 min. Relative light units (RLUs) were measured using a 20/20-n Single Tube Luminometer from Turner BioSystems (Sunnyvale). Assays were conducted in triplicate for each condition. Cell monolayers were washed with warm PBS. 0.75 mL of trypsin was added to each well, and 0.75 mL of MEM was added after complete trypsinization (trypsinization was monitored by light microscopy). Each sample was thoroughly resuspended and aliquoted into a plastic cuvette and the cell number immediately quantitated by determining the optical density at 800 nM using a spectrophotometer.

ELECTRON MICROSCOPY AND IMMUNOFLUORESCENCE

HeLa cells grown to 90% confluency in 6-well plates were infected with *C. pneumoniae*, pre-incubated with linear CdsN peptide for 30 min or with linear random peptide (MTS–MFAVNAQ) for 30 min at various concentrations, centrifuged for 45 min at room temperature, followed by incubation at 37°C for 1 h. The inoculum and peptide were removed and the medium replaced with MEM containing 1 μg/mL cycloheximide to inhibit host cell protein synthesis. Cells were incubated at 37°C for 72 h, collected by trypsinization and centrifugation at 1000 rpm for 10 min then fixed overnight at 4°C with 2% glutaraldehyde (v/v) in cacodylate buffer, 7.2. The cell pellets were post-fixed in 2% osmium tetroxide (v/v), embedded in Spurs medium, and thin sections were cut on a Reichert Ultracut E microtome. Sections were examined in a Jeol 1200 electron microscope at 80 kV and pictures were captured with an AMT digital camera. For immunofluorescent staining of inclusions, HeLa cells were infected as above using cell monolayers on glass cover slips in shell vials, fixed in methanol, and stained using the Pathfinder kit, which uses a FITC-conjugated major outer membrane protein (MOMP)-monoclonal antibody specific to *Chlamydia*, as per the manufacturer's instructions.

RESULTS

REGULATION OF CdsN BY CdsL

Type III secretion ATPases are known to be enzymatically down-regulated by YscL orthologs (Blaylock et al., 2006). *C. pneumoniae* encodes a YscL ortholog, CdsL (*Cpn0826*), which led us to explore whether CdsL down-regulates CdsN in *C. pneumoniae*. We have previously confirmed that CdsN interacted with CdsL using a GST pull-down assay, showing that CdsL co-purified with CdsN in 500 mM NaCl (Stone et al., 2008). We also showed previously that a CdsN-GST fusion protein possessed enzymatic activity consistent with other T3S ATPases (Stone et al., 2008). We found that CdsN hydrolyzed ATP at a rate of 0.0734 ± 0.0053 nmol/min at the concentration used in this assay (150 ng total). Prior to testing the ability of CdsL to regulate CdsN activity, we confirmed that CdsL lacked any ATPase activity by assaying it alone in the malachite green assay to control for possible contamination and found that CdsL possessed no ATPase activity. To test for regulation of CdsN by CdsL we added increasing amounts of CdsL to the CdsN reaction mixture. We found that addition of CdsL reduced CdsN enzymatic activity at a 0.3 CdsL:1 CdsN molar ratio, but the effect was most dominant at a 2.1 CdsL:1 CdsN molar ratio of CdsL: CdsN, where enzymatic activity was reduced by 82% (**Figure 1**). Further increasing the CdsL concentration only marginally reduced activity. The addition of a control His-tagged protein (His-CopN, a T3S structural component) had no effect on CdsN activity. Also as a control, His–CdsL had no effect on the enzymatic activity of a second *C. pneumoniae* ATPase, GspE (data not shown). GspE is a type II secretion ATPase which possesses enzymatic activity.

PEPSCAN ANALYSIS OF CdsN–CdsL INTERACTION

We have shown that CdsN is enzymatically down-regulated by CdsL, but the mechanism by which this occurs is unknown. In an attempt to expand our understanding of how CdsL may function to down-regulate CdsN, we have utilized Pepscan epitope mapping to identify the domains within CdsN responsible for the CdsL interaction. Purified His–CdsL was added to an over-lapping peptide library consisting of 4000 over-lapping linear and conformational peptides of the CdsN protein in search for the peptide domain mediating protein binding (Timmerman et al., 2007). CdsN contained two binding regions that mediated the interaction with CdsL. The two CdsL binding sequences spanned residues 221–229, and residues 265–270 corresponding to the amino acids "RSVIVVSTS" and "TRFARA," respectively, both of which are present within the catalytic domain (**Figure 2**). To corroborate the Pepscan data, we cloned and expressed a fragment of CdsN spanning residues 221–270 containing both of the CdsL predicted binding domains to test whether this CdsN fragment bound to His–CdsL. We found that the $CdsN_{221–270}$ co-purified with His–CdsL in the presence of 500 mM NaCl while GST alone did not co-purify with CdsL under any salt conditions (**Figure 3**).

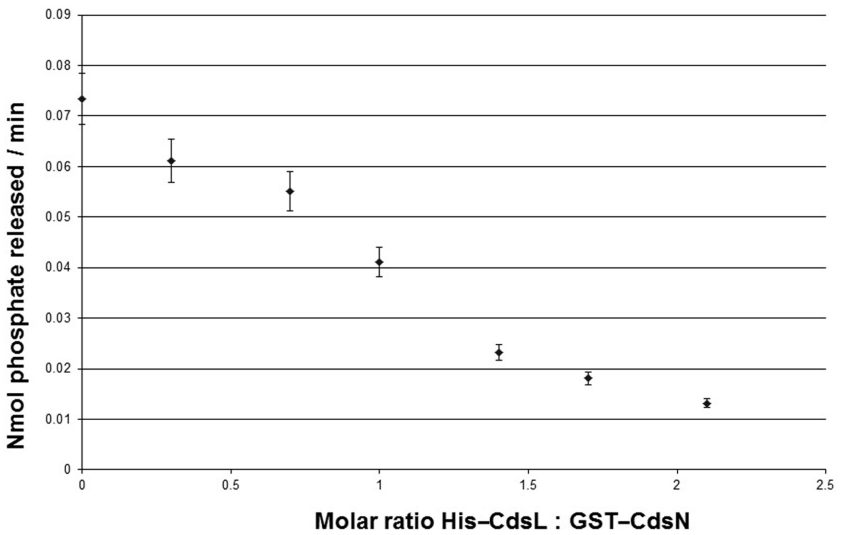


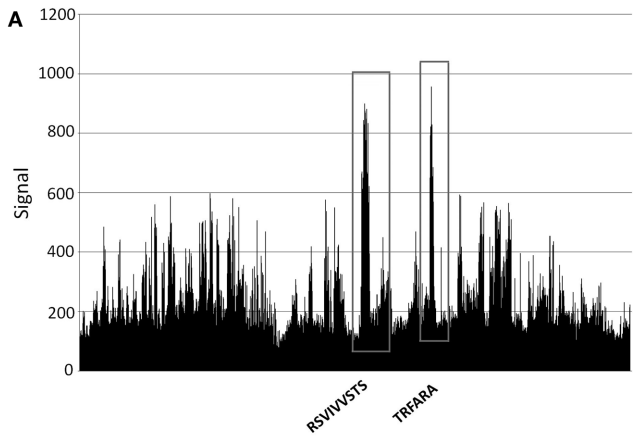
FIGURE 1 | CdsL down-regulations CdsN enzymatic activity in a dose-dependent fashion. Aliquots (150 ng) of GST–CdsN were assayed for enzymatic activity using the malachite green assay to detect released inorganic phosphate from ATP. His–CdsL was added to the reaction mixture at molar ratios between 0.3 and 2.1, and ATPase activity of GST–CdsN was measured. Increasing amounts of CdsL resulted in a decrease in CdsN enzymatic activity. The maximum reduction of activity was seen at a 2.1: CdsL:1 CdsN molar ratio (82% reduction in CdsN enzymatic activity). All experiments were performed in triplicate and data is presented as the mean with error bars representing 1 SD of the mean.

3D MODELING OF CdsN–CdsL BINDING DOMAIN

The crystal structure of the *E. coli* T3S ATPase, EscN, has recently been elucidated, allowing us to use an orthology modeling approach to obtain a predicted 3D structure of CdsN and to visualize the CdsL binding domains (Zarivach et al., 2007). CdsN and EscN possess a large amount of sequence orthology within the catalytic domain, where the CdsL binding domains are located (Stone et al., 2008). Using 3D-JIGSAW, a 3D structure of CdsN was generated and used to model the binding domains for CdsL. Modeling of the binding regions of CdsL on the predicted CdsN monomer revealed that both of the binding domains for CdsL are present in the central region of CdsN (**Figure 4A**). A more revealing mapping of the binding domains is shown when hexameric CdsN is modeled from the EscN crystal structure (Zarivach et al., 2007). For clarity, we have shown only one half of the predicted hexameric structure of CdsN (a CdsN trimeric complex). Residues 221–229 are exposed toward the outer face of the hexamer (**Figure 4B**, blue), while residues 265–270 are exposed in the central channel of the hexameric ring (**Figure 4C**, red). The location of the binding domains is consistent with a proposed mechanism of CdsL down-regulation of CdsN sterically hindering access of ATP to the P-loop (**Figures 4B,C**, magenta).

INHIBITION OF *C. PNEUMONIAE* GROWTH AND REPLICATION BY CdsN COGNATE PEPTIDE

The Pepscan binding data revealed two distinct domains of CdsN that mediate the interaction between CdsN and CdsL. Using this information we sought to design and utilize a peptide mimetic targeting the interaction between CdsN and CdsL and examine its effect on chlamydial replication. As discussed above, CdsN possessed two different binding domains for CdsL; we selected the six amino acid sequence TRFARA to create a potential peptide mimetic. We synthesized the TRFARA peptide with a N-terminal MTS, YGRKKRRQRRR (Efthymiadis et al., 1998). We also extended the CdsN sequence flanking the TRFARA peptide to ensure the entire binding domain was included, and flanked the peptide with cysteine residues to assist with any disulfide bonding that may be required for binding. The final peptide utilized was the 28-amino acid sequence YGRKKRRQRRRCVVLMMDSVTRFARALC. To examine whether the peptide-MTS was entering EBs, we first incubated either GST alone or GST–MTS at 50, 100, and 500 μM with gastrografin purified EBs. We saw that the amount of GST–MTS entering EBs increased in a dose-dependent manner (**Figure 5A**). The GST control (500 μm) was taken up poorly by *Chlamydia*. We then added either GST or GST–MTS for increasing time periods (5, 30, or 60 min) which showed increasing uptake with time, starting as early as 5 min (**Figure 5B**). Again, the GST control showed very poor uptake into *Chlamydia*, suggesting that the MTS facilitates peptide entry in a time- and dose-dependent manner. HeLa cells were then infected with *C. pneumoniae* at an MOI of 2 and stained for inclusions at 72 h using a FITC-conjugated anti-MOMP monoclonal antibody. The CdsN peptide was added to EBs at a 0, 20, 50, and 100-μM concentrations for 30 min prior to infection and we saw that infectivity was reduced in a dose–response fashion (**Figure 6A**). In the absence of the peptide, inclusions are clearly visible at 72 h (**Figure 7**). Pre-treatment of the cells with 50 μM peptide resulted in a drastic reduction in the number of inclusions (>95%), while at 100 μM there was toxicity for HeLa cells. To rule out the possibility that the inhibitory activity at 50 μM might be due to toxicity we tested the effect of the peptide at 50 μM on HeLa cells using an adenylate kinase release assay and found little or no toxicity (**Figure 6B**). We next examined infected cells with the CdsN or the random peptide by transmission electron microscopy. In infected cells exposed to the random peptide large inclusion bodies containing a mixture of RBs and EBs were readily visible



B

CdsN:
MDQLTTDFDTLMSQLGDVNLTTVVGRITEVVGMLIKAVVPNVRVGEV
CLVKRNGMEPLVTEVVGFTQSFAFLSPLGELSGVSPSSEVIPTGLPLHIRA
GNGLLGRVLNGLGEPIDVETKGPLQNVDQTFPIFRAPPDPLHRAKLRQI
LSTGVRCIDGMLTVARGQRIGIFAGAGVGKSSLLGMIARNAEEADVNVI
ALIGERGREVREFIEGDLGEEGMK**RSVIVVSTS**DQSSQLRLNAAYVGTAI
AEYFRDQGKTVVLMMDSV**TRFARA**LREVGLAAGEPPARAGYTPSVFST
LPRLLERSGASDKGTITAFYTVLVAGDDMNEPVADEVKSILDGHIVLSNA
LAQAYHYPAIDVLASISRLLTAIVPEEQRRIIGKAREVLAKYKANEMLIRIG
EYRRGSDREIDFAIDHIDKLNRFLKQDIHEKTNYEEAAQQLRAIFR

FIGURE 2 | Pepscan analysis of the CdsL binding domain on CdsN. An over-lapping peptide library for full length CdsN consisting of 4000 linear and looped peptides was constructed by Pepscan Presto (The Netherlands). His–CdsL was screened against the peptide library to identify CdsN peptides that interact with CdsL. **(A)** Raw Pepscan data obtained by screening full length His–CdsL against the over-lapping peptide library of 4000 linear and looped CdsN peptides (*x*-axis). The two distinct CdsL binding domains are highlighted in black within the vertical boxes, and the signal strength of the interaction is visualized on the *y*-axis. The two peaks correspond to the sequences RSVIVVSTS and TRFARA. **(B)** The two amino acid sequences to which CdsL binds (RSVIVVSTS and TRFARA) are bolded within the full length CdsN amino acid sequence.

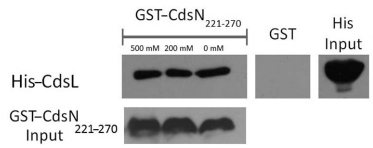


FIGURE 3 | The CdsN$_{221–270}$ peptide, containing both the predicted CdsN–CdsL binding domains, co-purifies with CdsL. GST–CdsN$_{221–270}$ was bound to glutathione beads and used to pull-down His–CdsL from *Escherichia coli* lysates. Beads were collected by centrifugation, washed with 0, 200, and 500 mM NaCl and probed for His-tagged protein by Western blot using anti-His antibody. GST–CdsN$_{221–270}$ co-purified with CdsL while GST alone did not.

while infected cells incubated with 50 μM CdsN peptide lacked any detectable inclusions when over 200 cells were examined and showed no evidence of cytotoxicity (**Figure 7**).

DISCUSSION

Although *C. pneumoniae* contains all the genes coding for a T3SS, only a small number of these have been characterized. We have previously shown that CdsN possesses enzymatic activity and interacts with the putative type III secretion protein, CdsL (Stone et al., 2008). We have now extended these observations to show that CdsL functions to down-regulate CdsN enzymatic activity. We have also used a novel approach to map the exact region within CdsN that mediates the CdsN–CdsL interaction, and used this information to design a peptide mimetic that disrupts the chlamydial replication cycle. Combined, this data provides new insights into the regulation of the T3S system and its importance in the chlamydial life-cycle.

YscL orthologs have been shown to down-regulate T3S ATPase enzymatic activity in *Yersinia*, and *C. pneumoniae* encodes a YscL ortholog (CdsL; Blaylock et al., 2006; Stone et al., 2008). We found that CdsN treated with increasing concentrations of CdsL reduced enzymatic activity up to 82%. To show that the regulation by CdsL was specific we tested the ability of CdsL to down-regulate the type II secretion ATPase of *Chlamydia*, GspE, and found that it had no effect on its enzymatic activity. This suggests that CdsL is specific in its down-regulation of CdsN. CdsL has previously been shown to be expressed at all time points throughout the replication cycle with an apparent accumulation during the final 48 h (Slepenkin et al., 2003). This is consistent with a role for CdsL regulating T3S during invasion and intracellular replication phases of the life-cycle in EBs and RBs, respectively.

Type III secretion ATPases are known to mediate numerous protein interactions between both structural components and chaperone/effector complexes, and play a critical role in construction of the injectisome and secretion of effectors (Gauthier and Finlay, 2003; Akeda and Galan, 2005). We used Pepscan Analysis to determine the CdsN domain that mediates the interaction with CdsL. We found that there were two distinct domains within CdsN that interacted with CdsL; one exposed in the central channel of the hexameric structure of CdsN and the other exposed on the outer face. Using GST pull-downs, we corroborated the Pepscan data by demonstrating that a 49-amino acid fragment of CdsN containing both of the predicted CdsL binding domains was able to co-purify with full length CdsL. We have shown previously that CdsL forms dimers, and it is likely that the two CdsL molecules interact with different regions of CdsN (Pallen et al., 2006; Stone et al., 2008). This would be consistent with the two binding domains on CdsN. Along with regulating ATPase activity, CdsL is believed to tether the ATPase to the inner membrane, functioning in a similar manner as the gamma-stalk of the F_0F_1 ATPase which runs through the central channel of the F_0F_1 hexamer and may interact with the membrane (Pallen et al., 2006). The location of the CdsL binding domain within the central channel of CdsN supports the concept that CdsL functions in a similar manner as the gamma-stalk domain for CdsN. Both binding domains are found in close proximity to the P-loop within the catalytic domain which coordinates ATP binding and cleavage (Stone et al., 2008). It is possible that when CdsL is bound to either of these domains it could sterically hinder access of ATP to the P-loop, reducing enzymatic function of CdsN. The tethering function of CdsL would be crucial for T3S function, and if this tethering is mediated by the binding domain within the central channel of the CdsN hexamer then likely this interaction is maintained throughout the entire developmental cycle. The exposed binding domain on the outer surface of the CdsN hexamer, however, could be responsible for the down-regulation of CdsN by CdsL. CdsL could

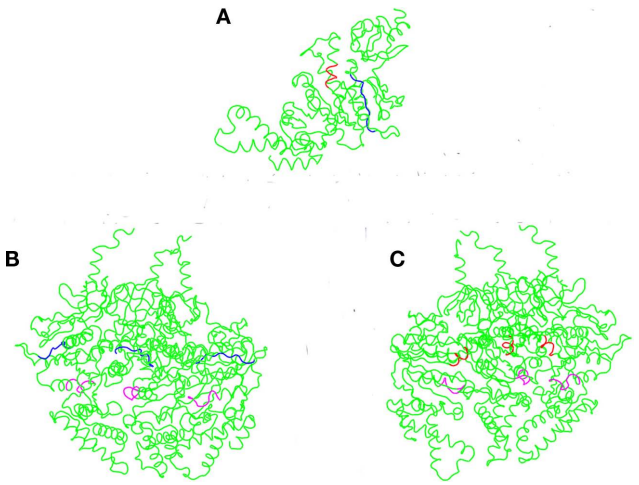


FIGURE 4 | CdsL binding domains mapped onto the predicted 3D structure of CdsN reveals two distinct domains. A 3D structure of CdsN was generated based on orthology modeling using 3D-JIGSAW based on the structure of EscN, and used to visualize the CdsL binding domains identified from – 30 – the peptide library binding data. The two CdsL binding domains on CdsN are in blue (residues 221–229) or red (residues 265–270), and the ATP coordinating P-loop region of CdsN is highlighted in magenta. **(A)** Representation of the predicted CdsN monomeric structure indicating both CdsL binding domains (RSVIVVSTS in blue, TRFARA in red). **(B)** Structural model showing the location of residues 221–229 (RSVIVVSTS), displayed on one half of the predicted hexameric structure and viewed from the outer face, revealing that the binding domains are exposed to the outside of the hexamer. The P-loop is represented in magenta. **(C)** Structural model viewed from outside looking toward the central channel showing the location of residues 265–270 (TRFARA), displayed on one half of the predicted hexameric structure revealing that the domain is exposed within the central channel. The P-loop is represented in magenta.

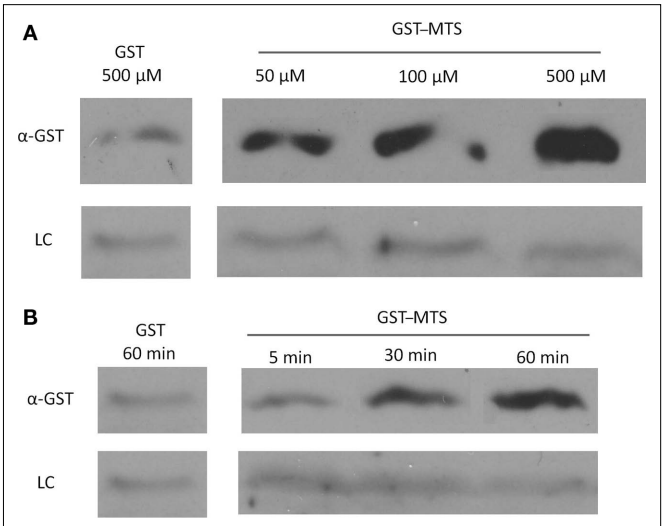


FIGURE 5 | Uptake of a GST-Membrane transport signal (MTS) fusion protein into EBs. EBs were purified on a discontinuous gastrografin gradient and collected by centrifugation to ensure the use of a homogenous EB mixture. The GST alone and GST–MTS protein was expressed in *Escherichia coli* cells and purified using glutathione beads. The EBs were then incubated with either GST or GST–MTS, trypsinized for 30 min to ensure that all extracellular GST or GST–MTS was degraded, and examined by α-GST Western blot for the presence of intracellular GST-tagged protein. CdsL, an intracellular type III secretion protein, was used as a loading control (LC). **(A)** Time-course for incubation of EBs with GST–MTS for 5, 30, or 60 min. GST control at 60 min displayed very little protein while GST–MTS at 5, 30, and 60 min had increasing amounts of GST–MTS present within the EBs. **(B)** Dose–response of EBs incubated with GST–MTS at 50, 100, and 500 µm. GST control at 500 µm displayed very little protein while GST–MTS accumulated within the EBs as the concentration increased. CdsL is shown as a LC.

bind and dissociate from the outer CdsN–CdsL binding domain upon a conformational change in CdsN, thereby activating ATPase activity upon CdsL release by allowing ATP to access the P-loop. It is tempting to speculate that delivery of effector/chaperone complexes to CdsN, possibly by a multi-cargo transport protein such as CdsQ, could be the trigger for CdsL release and ATPase activation, allowing for the dissociation of the effector/chaperone complex and subsequent secretion of the effector (Spaeth et al., 2009).

Using Pepscan epitope mapping we have shown where CdsL binds to CdsN. Using this binding data we designed a cognate CdsN peptide containing a membrane transport signal to allow the peptide to enter *Chlamydia* and showed that this peptide blocked chlamydial invasion of HeLa cells. Membrane transport signals are known to interact hydrophobically with eukaryotic membranes, triggering entry by endocytosis across the membrane (Efthymiadis et al., 1998). Our working hypothesis is that this transport signal would facilitate entry of the CdsN peptide into EBs, and that this could potentially disrupt the CdsN–CdsL interaction and inhibit T3S, and thus invasion, although we have not shown inhibition of T3S *per se*. This could be achieved by either disrupting the regulation of the ATPase or interfering with the tethering function of the ATPase, displacing its peripheral association with the inner membrane. Either of these two scenarios could disrupt the T3SS, preventing chlamydial infection and cellular invasion. We first confirmed that the MTS facilitated uptake of the peptide into EBs in a time- and dose-dependent manner. Afterward, we treated the chlamydial EBs for 30 min with the CdsN peptide which reduced chlamydial infection in a dose–response fashion, and at a concentration of 50 µM reduced *Chlamydia* infectivity by >95%, as determined by IF staining and counting of inclusions at 72 h. An alternative explanation of this data could be that the peptide was bactericidal toward *Chlamydia*, but we feel that this is unlikely since a random peptide failed to inhibit chlamydial replication. We have also shown that two additional peptide mimetics targeting other essential protein interactions can also inhibit chlamydial replication (Mahony et al., 2010). We are currently investigating whether the CdsN peptide mimetic can inhibit chlamydial replication if administered during the infection cycle, once infection has occurred, which if successful would indicate that the peptide can disrupt CdsN–CdsL complexes within RBs and block T3S across the inclusion membrane. This would suggest that ATPase activity is carefully regulated by CdsL in both EBs and RBs, and the CdsN–CdsL interaction is critical for chlamydial replication.

The discovery of unique CdsN binding domains for CdsL coupled with structural modeling of the binding domains has allowed us to postulate how CdsL functions to down-regulate CdsN. This has allowed us to create a cognate CdsN peptide that functions as a novel antimicrobial agent, preventing *C. pneumoniae* from

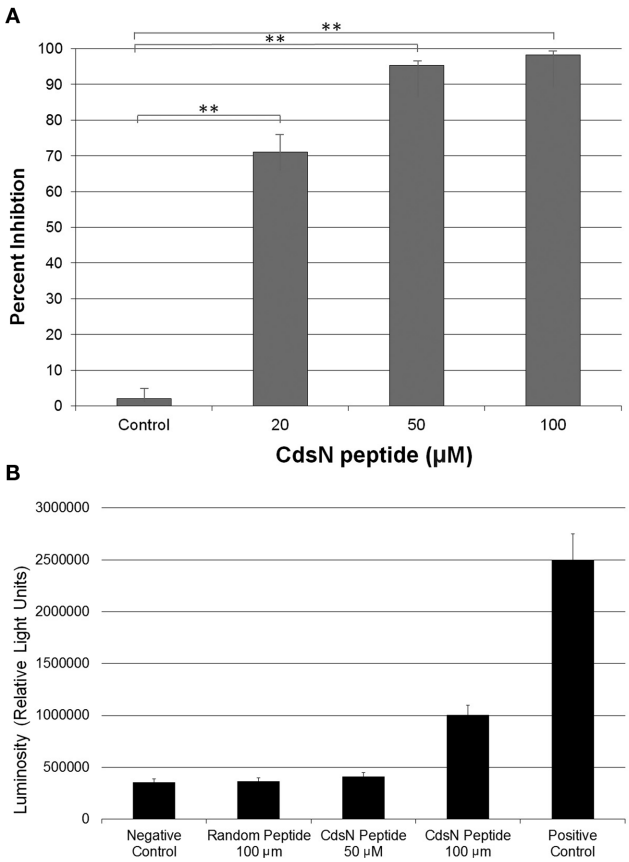


FIGURE 6 | CdsN peptide containing the CdsL binding sequence TRFARA inhibits the infection of HeLa cells in a dose-dependent fashion and shows low toxicity at 50 μM. Shell vials grown to 85% confluency were infected with *Chlamydia pneumoniae* at an MOI of 3 after incubation with the CdsN or control peptide. Infected cells were counted by staining with a *Chlamydia* specific major outer membrane protein (MOMP)-monoclonal antibody (Pathfinder kit). Toxicity was measured using an adenylate kinase release assay for HeLa cells in the presence of either the CdsN or control peptide. **(A)** CdsN peptide was added at increasing concentrations (20, 50, and 100 μM) and demonstrated a dose–response in reduction of *C. pneumoniae* infectivity of HeLa cells. The control represents a random peptide with the sequence MFAVNAQ–MTS that had no effect on *C. pneumoniae* infectivity. All experiments were performed in triplicate and data is presented as the mean with error bars representing the mean + 1 SD with **representing $p < 0.01$. **(B)** Toxicity of the CdsN peptides toward HeLa cells was evaluated using the Lonza adenylate kinase release assay. The CdsN peptide was added to HeLa cells for 1 h at 50 and 100 μM, and the random peptide was added for 1 h at 100 μM. We saw that the CdsN peptide at 50 μM had no toxicity toward HeLa cells, and only minor toxicity at 100 μM.

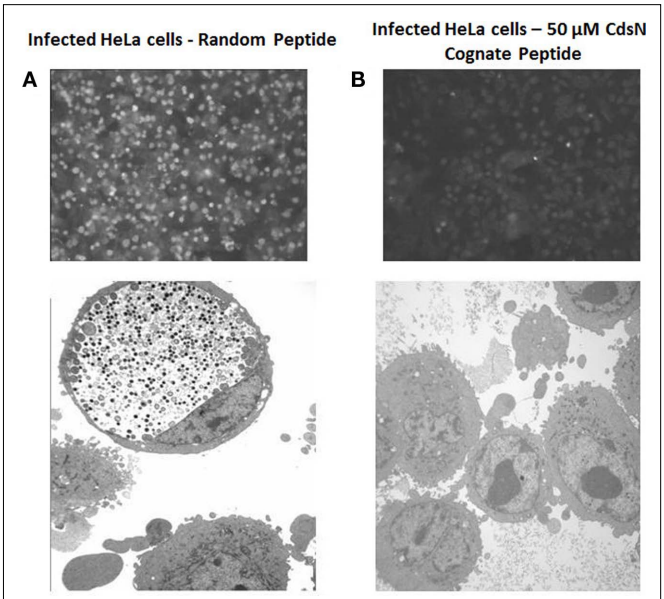


FIGURE 7 | CdsN peptide reduces chlamydial infectivity of HeLa cells by IF staining and EM. HeLa cells were pretreated with CdsN or a control peptide (MTS–MFAVNAQ), then infected with *Chlamydia pneumoniae* at an MOI of 3 and examined by either IF staining using anti-MOMP monoclonal antibody treatment with the pathfinder kit (top) or by electron microscopy (bottom). **(A)** HeLa cells treated with the control peptide (100 μM) and infected with *C. pneumoniae*. Immunofluorescent staining reveals numerous infected cells (top) while electron microscopy reveals a large inclusion body with both EBs and RBs (bottom). **(B)** HeLa cells treated with the CdsN peptide (50 μM) and infected with *C. pneumoniae*. Evaluation of over 200 cells treated with the CdsN peptide by IF staining and EM examination revealed a drastic reduction in *C. pneumoniae* infected cells and a complete lack of detectable inclusions by EM.

invading host cells. One of the major difficulties in working with *Chlamydia* is that they are genetically intractable. Peptide mimetics such as this CdsN peptide could effectively create chemical knockouts by disrupting protein complexes, allowing us to mimic genetic knockouts and explore specific functions of various proteins in the T3SS.

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Chlamydia trachomatis secretion of proteases for manipulating host signaling pathways

Guangming Zhong*

Department of Microbiology and Immunology, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA

Edited by:
Robert Heinzen, NIH/NIAID-RML, USA

Reviewed by:
Marci Scidmore, Cornell University, USA
Dan Rockey, Oregon State University, USA

***Correspondence:**
Guangming Zhong, Department of Microbiology and Immunology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229, USA.
e-mail: zhongg@uthscsa.edu

The human pathogen *Chlamydia trachomatis* secretes numerous effectors into host cells in order to successfully establish and complete the intracellular growth cycle. Three *C. trachomatis* proteases [chlamydial proteasome/protease-like activity factor (CPAF), tail-specific protease (Tsp), and chlamydial high temperature requirement protein A (cHtrA)] have been localized in the cytosol of the infected cells either by direct immunofluorescence visualization or functional implication. Both CPAF and Tsp have been found to play important roles in *C. trachomatis* interactions with host cells although the cellular targets of cHtrA have not been identified. All three proteases contain a putative N-terminal signal sequence, suggesting that they may be secreted via a sec-dependent pathway. However, these proteases are also found in chlamydial organism-free vesicles in the lumen of the chlamydial inclusions before they are secreted into host cell cytosol, suggesting that these proteases may first be translocated into the periplasmic region via a sec-dependent pathway and then exported outside of the organisms via an outer membrane vesicles (OMVs) budding mechanism. The vesiculized proteases in the inclusion lumen can finally enter host cell cytosol via vesicle fusing with or passing through the inclusion membrane. Continuing identification and characterization of the *C. trachomatis*-secreted proteins (CtSPs) will not only promote our understanding of *C. trachomatis* pathogenic mechanisms but also allow us to gain novel insights into the OMV pathway, a well-known mechanism used by bacteria to export virulence factors although its mechanism remains elusive.

Keywords: *Chlamydia trachomatis*, proteases, secretion, pathogenesis

INTRODUCTION

Chlamydia trachomatis is the most frequently reported bacterial sexually transmitted infection in the US (Centers for Disease Control Prevention, 2009), which, if untreated, can lead to severe complications characterized with inflammatory pathologies, including pelvic inflammatory diseases, ectopic pregnancy, and infertility (Land et al., 2010). The chlamydial intracellular replication is thought to significantly contribute to the *C. trachomatis*-induced inflammatory pathologies (Stephens, 2003; Wyrick, 2010). A typical chlamydial replication cycle starts with the invasion of an epithelial cell with a chlamydial infectious elementary body (EB), which is facilitated by chlamydial injection of preexisting effectors into the epithelial cell to induce and modulate endocytosis (Clifton et al., 2004, 2005; Engel, 2004; Hower et al., 2009). Once internalized, an EB differentiates into a non-infectious but metabolically active reticulate body (RB). The RB makes new proteins not only for multiplication but also for secretion into the inclusion lumen and membrane (Rockey et al., 1995, 2002; Luo et al., 2007a,b,c; Li et al., 2008) as well as host cell cytosol (Valdivia, 2008; Zhong et al., 2009, 2011; Betts-Hampikian and Fields, 2010) via a type III secretion (T3S, Fields and Hackstadt, 2000; Valdivia, 2008; Betts-Hampikian and Fields, 2010), sec-dependent secretion (Chen et al., 2010b), or an autotransporter (Henderson and Lam, 2001; Wehrl et al., 2004; Carlson et al., 2005; Vandahl et al., 2005; Kiselev et al., 2009; Byrne, 2010) pathways. After replication, the progeny RBs differentiate back into EBs for spreading to near-by cells. It is thought that the *C. trachomatis*-secreted proteins (CtSPs; ref: Valdivia, 2008; Zhong, 2009, 2011; Betts-Hampikian and Fields, 2010) are not only necessary for completing the existing developmental cycle but also essential for ensuring a successful start of subsequent infection cycles. Identification and characterization of CtSPs may provide important knowledge for understanding chlamydial pathogenic mechanisms and improving diagnosis, treatment, and prevention of *C. trachomatis* infection. Thus, identification of CtSPs has become an intensively investigated topic for chlamydiaologists.

CHLAMYDIA TRACHOMATIS-SECRETED PROTEASES IN PATHOGENESIS

A *C. trachomatis* genome typically encodes more than two dozens of proteins with proteolytic activity (Stephens et al., 1998). A function-driven approach has led to the identification of a novel serine protease, designated as chlamydial proteasome/protease-like activity factor (CPAF; Zhong et al., 2001). The chlamydial proteases are usually distributed in the chlamydial cytosol, periplasmic region and/or various membranes for fulfilling their respective roles in chlamydial biology. However, some, including CPAF (encoded by ORF CT858), the tail-specific protease (Tsp, CT441), and the chlamydial high temperature requirement protein A protease (cHtrA, CT823), are also found outside of the chlamydial organisms by direct visualization and/or functional implication, suggesting that these proteases may be used by *C. trachomatis* organisms to target host proteins for manipulating host signaling pathways.

Chlamydial proteasome/protease-like activity factor is an extensively studied Chlamydia-secreted serine protease with a water molecule-mediated catalytic triad consisting of residues H105, S499, and E558. CPAF and it can undergo autoprocessing for activation (Dong et al., 2004a,b; Huang et al., 2008; Chen et al., 2009, 2010a). CPAF has a broad substrate specificity and attacks a wide spectrum of host proteins, including the transcriptional factors USF-1 (Zhong et al., 1999) and RFX5 (Zhong et al., 2000) for potentially evading immune recognition, and HIF-1 (Rupp et al., 2007) for dealing with stress responses, the BH3-only proteins (proapoptotic members of the Bcl-2 family; Fischer et al., 2004; Dong et al., 2005; Pirbhai et al., 2006) for inhibiting apoptosis (Fan et al., 1998; Greene et al., 2004; Xiao et al., 2004, 2005; Zhong et al., 2006), the DNA repairing enzyme PARP [Poly (ADP-ribose) polymerase], and cell cycling proteins (Balsara et al., 2006; Paschen et al., 2008) for altering cell cycle, cytoskeleton proteins (keratins 8 and 18, vimentin Dong et al., 2004c; Kumar and Valdivia, 2008; Savijoki et al., 2008) for promoting inclusion expansion, and even cell surface proteins CD1d (Kawana et al., 2007) and nectin-1 (Sun and Schoborg, 2009). Although the functional consequences of some of the host protein degradation by CPAF remain unknown, the host protein degradation should benefit *C. trachomatis* intracellular growth (Zhong, 2009).

Although Tsp was not detected outside of inclusions using standard immunofluorescence assays (data not shown), it was found to cleave host NF-κB in *C. trachomatis*-infected cells (Lad et al., 2007a,b), suggesting that an undetectable amount of Tsp might be secreted into host cell cytosol for interrupting NF-κB function. During *C. trachomatis* infection, there were conflicting observations: On one hand, *C. trachomatis* infection activated a wide variety of inflammatory cytokines, including IL-1, IL-6, IL-8, and TNFα (Rasmussen et al., 1997; Cheng et al., 2008); On the other, no significant NF- κB activation was detected in the infected cells (Xiao et al., 2005; Lad et al., 2007a). It turned out that the *C. trachomatis* organisms used the MAP kinase pathway to promote chlamydial acquisition of host lipids, during which inflammatory responses were inevitably activated (Su et al., 2004). The lack of NF-κB activation was probably due to the chlamydial ability to actively silence the NF- κB inflammatory pathway. NF- κB p65 was cleaved into two major fragments (p40 and p20) by Tsp (Lad et al., 2007a) and CPAF (Christian et al., 2010). Since the N-terminal fragment p40 maintained the ability to interact with I-κBα (a cytoplasmic inhibitor of NF-κB) and to bind to DNA but lacked transactivation capability, the p40 might be able to block the residual full length p65-mediated response via a dominant negative effect (Lad et al., 2007a). Tsp was also reported to interact with the host SRAP1 co-activator of estrogen receptor α (Borth et al., 2010). However, the biological significance of chlamydial Tsp-host SRAP-1 interaction remains unknown.

We recently detected the *C. trachomatis* periplasmic protease cHtrA in both the chlamydial inclusion lumen and host cell cytosol (**Figure 1**), suggesting that the chlamydial periplasmic protein cHtrA is also secreted into host cells. The secretion appeared to be specific since no other chlamydial periplasmic proteins including CT067 (Miller et al., 2009) were detected outside of the chlamydial inclusions (**Figure 1** and data not shown). Normally, HtrA from eukaryotic and prokaryotic species exhibits both chaperone and proteolytic activities with a broad proteolytic substrate specificity (Huston et al., 2007, 2008). HtrA is a hexamer formed by staggered association of trimeric rings and the access to the proteolytic sites in central cavity is controlled by 12 PDZ domains in the sidewall (Krojer et al., 2002, 2010). In eukaryotic cells, HtrA responds to unfolded proteins in the endoplasmic reticulum (ER) by cleaving and releasing the ER membrane-anchored transcription factors ATF6 and SREBP into nucleus to activate the expression of proteins required for the unfolded protein response and cholesterol biosynthesis (Brown and Goldstein, 1999; Ye et al., 2000). In bacteria, the periplasmic HtrA, in response to the binding of C-terminal peptides from unfolded/reduced outer membrane proteins, cleaves, and releases the σ^E-factor to activate stress response genes (Walsh et al., 2003). Since HtrA is required for bacterial survival under high temperature, it is called High temperature requirement (Htr) protein (Missiakas et al., 1997). Although both the tertiary structure and function of HtrA are well-known, the role of cHtrA in chlamydial pathogenesis remains unclear. The finding that cHtrA was localized both in the chlamydial inclusion lumenal space and the host cell cytosol suggests that the chlamydial periplasmic cHtrA may also contribute to the chlamydial proteolysis strategies for manipulating host cell signaling pathways. However, it is still unknown how the secreted cHtrA contributes to chlamydial pathogenesis. Can the secreted cHtrA gain access to host cell ER to regulate the host unfolded protein stress responses? What are the cellular targets of the secreted cHtrA during chlamydial infection? Interestingly, HtrA from the human gastric pathogen *Helicobacter pylori* is also secreted outside the bacteria (Lower et al., 2008). More importantly, it can cleave E-cadherin to disrupt epithelial tight junction, which may benefit the bacterial invasion of the gastric epithelial tissues (Hoy et al., 2010). Since HtrA and other conserved proteases are

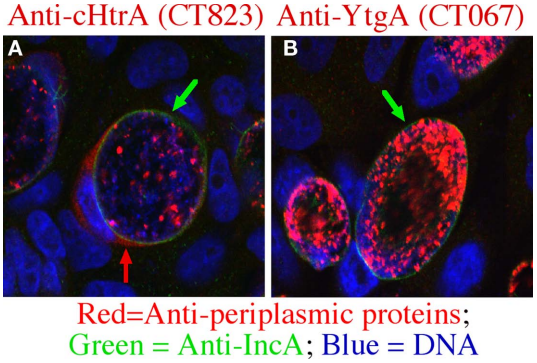

FIGURE 1 | Chlamydial high temperature requirement protein A (cHtrA) is selectively secreted into host cell cytosol. The *Chlamydia trachomatis*-infected HeLa cells grown on coverslips were processed at 36 h after infection for an immunofluorescence assay as described previously (Zhong et al., 1997). The samples were triply labeled with mouse anti-cHtrA [CT823 **(A)**] or YtgA [CT067 **(B)**] antibodies visualized with a goat anti-mouse IgG conjugated with Cy3 (red), a rabbit anti-IncA antibody visualized with a goat anti-rabbit IgG conjugated with Cy2 (green), and the DNA dye Hoechst (blue). The images were acquired using an Olympus confocal microscope. Green arrows indicate inclusion membrane while the red arrow indicates cHtrA secreted into host cell cytosol. Note that only cHtrA but not YtgA was detected outside of the inclusion membrane although both are considered chlamydial periplasmic proteins (Miller et al., 2009).

known to play important roles in bacterial pathogenesis (Ingmer and Brondsted, 2009), identifying cellular targets of the Chlamydia-secreted cHtrA should provide novel insights into chlamydial pathogenic mechanisms.

POTENTIAL PATHWAYS REQUIRED FOR *C. TRACHOMATIS* SECRETION OF PROTEASES INTO HOST CELL CYTOSOL

All three proteases localized in the host cell cytosol contain an N-terminal signal sequence, suggesting that they may be translocated into the periplasmic region via a sec-dependent secretion pathway. Among the many known secretion pathways, the sec-dependent or twin-arginine translocon (Tat) pathway is used by bacteria to deliver proteins into the periplasmic space. The Tat translocase, consisting of the TatA/E, B and C proteins, is responsible for transporting folded proteins across the inner membrane (Dilks et al., 2003; Lee et al., 2006). However, *C. trachomatis* genome does not encode any homolog of the Tat translocases (Stephens et al., 1998) but encodes all essential components required for a functional sec-dependent pathway (Stephens et al., 1998), suggesting that *C. trachomatis* organisms can use the universally conserved sec-dependent pathway to translocate proteins with an N-terminal signal sequence from cytoplasm into periplasmic regions. Indeed, we have previously demonstrated that a sec-dependent pathway is required for exporting CPAF to host cell cytosol (Chen et al., 2010b). The N-terminal signal sequence of CPAF (CPAFss) was cleaved from mature CPAF and CPAFss directed translocation of PhoA into bacterial periplasm.

However, the sec-dependent pathway alone can only deliver its cargoes into the periplasmic region. The periplasmic proteins are further exported outside of the bacterial organisms using the chaperone/usher, autotransporter, or Type II outer membrane GspD pore complex pathways. Although chlamydial genome encodes homologs of both autotransporters and GspD, these pathways deliver periplasmic proteins out of the organisms in free form. It is hard to imagine how free proteins secreted into the inclusion lumen can pass through the inclusion membrane and enter host cell cytosol in a regulated/controlled manner. Interestingly, both CPAF (**Figure 2**) and cHtrA (data not shown) were detected in organism-free granules in the lumen of inclusions before their secretion into host cell cytosol. It is thus hypothesized that the chlamydial proteases that are translocated into the periplasmic regions may be further exported to the lumen of inclusions via an outer membrane vesicles (OMVs) budding mechanism. This hypothesis is supported by various previous observations that the chlamydial RB outer membrane was induced to undergo vesiculation (Matsumoto and Manire, 1970) and chlamydial organism-free vesicles were detected both inside (Jorgensen and Valdivia, 2008) and outside of inclusion membrane (Giles et al., 2006). The vesiculized proteases may further enter host cell cytosol by vesicle fusing with or passing through the inclusion membrane. Although OMVs have been recognized as an essential means for gram-negative bacteria to secrete virulence factors (Bomberger et al., 2009; Ellis and Kuehn, 2010; Parker et al., 2010; Unal et al., 2010), the precise mechanisms on how OMVs are regulated remain unknown (Haurat et al., 2011). That's why some remain skeptical about whether OMVs can represent a specific mechanism for protein secretion. The *C. trachomatis*-infected cells by allowing multiple vesiculized "effectors" to be studied simultaneously in a confined space may provide a unique opportunity for us to gain novel insights into the mechanisms of OMVs.

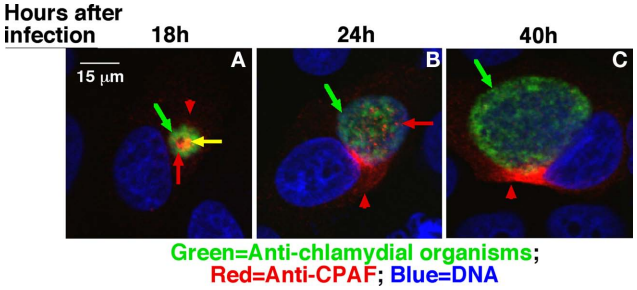

FIGURE 2 | Detection of CPAF in chlamydial organism-free vesicles in the lumen of chlamydial inclusions. The *Chlamydia trachomatis*-infected HeLa cells grown on coverslips were processed at 18 h **(A)**, 24 h **(B)**, or 40 h **(C)** after infection as indicated on top of the figure for an immunofluorescence assay as described in **Figure 1** legend except that the monoclonal antibody 100a against CPAF was used to replace the mouse antibodies and a rabbit anti-chlamydial organism antibody was used to replace the anti-IncA antibody. The images were also acquired using an Olympus confocal microscope. Green arrows indicate chlamydial organisms. Red arrows indicate CPAF-laden granules that are free of chlamydial organisms while the yellow arrow indicates CPAF that overlaps with chlamydial organisms. Red arrowheads indicate CPAF molecules secreted into host cell cytosol. Please note that at the early time points, CPAF was detected in granules in the inclusions and many of the CPAF-positive granules were free of chlamydial organisms in the 24 h sample.

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Cheating death: a *Coxiella* effector prevents apoptosis

Laura J. Broederdorf and Daniel E. Voth*

Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, AR, USA

**Correspondence: dvoth@uams.edu*

A commentary on

Inhibition of pathogen-induced apoptosis by a *Coxiella burnetii* type IV effector protein

by Luhrmann, A., Nogueira, C. V., Carey, K. L., and Roy, C. R. (2010). Proc. Natl. Acad. Sci. U.S.A. 107, 18997–19001.

Intracellular bacterial pathogens have developed strategies to subvert numerous host cell processes, often by deploying a battery of secreted proteins, termed effectors, into the cytosol. The stealthy agent of Q fever, *Coxiella burnetii*, continuously manipulates its eukaryotic host cell throughout a prolonged infectious cycle and replicates in a unique phagolysosome-like vacuole. The organism encodes a Dot/Icm type IV secretion system (T4SS) similar to that of closely related *Legionella pneumophila*, the causative agent of Legionnaires' disease (Seshadri et al., 2003). These pathogens' respective T4SSs are predicted to translocate a large number of effectors directly from the vacuole into the host cell cytosol where they interact with eukaryotic proteins to influence infection events. While over 300 *Legionella* Dot/Icm substrates have been identified (Hubber and Roy, 2010), *Coxiella* encodes few obvious homologs of these or other bacterial effectors, indicating the use of pathogen-specific repertoires. However, T4SS conservation allows the use of *Legionella* to study *Coxiella* effectors, expanding the panel of tools available for this genetically intractable organism.

Recent studies show *Coxiella* isolates encode 14 Ank proteins containing eukaryotic-like ankyrin repeat domains, 11 of which are translocated by the *Legionella* Dot/Icm T4SS (Pan et al., 2008; Voth et al., 2009). However, Ank function has remained a mystery until a recent report by Luhrmann et al. (2010) examining *Coxiella* effector-driven anti-apoptotic activity. *Coxiella* antagonizes intrinsic apoptotic death in macrophages (Voth et al., 2007) and prevents cytochrome *c* release from mitochondria to provide a stable intracellular niche for replication (Luhrmann and Roy, 2007). The pathogen also activates Akt and Erk1/2 signaling to promote survival (Voth and Heinzen, 2009). Each of these events relies on *Coxiella* protein synthesis, suggesting the organism secretes a distinct effector(s) to regulate apoptosis. Unfortunately, effector identification and characterization has been hampered by a lack of methods for *Coxiella* genetic manipulation. As an alternative, Luhrmann et al. (2010) investigated the mechanistic activity of a *Coxiella* Ank through exploration of its anti-apoptotic function in *Legionella*.

Luhrmann et al. (2010) tested the ability of four Anks (AnkA, B, F, and G) to inhibit intrinsic apoptosis. Eukaryotic cells ectopically expressing individual Anks were treated with the apoptosis-inducing agent staurosporine. Only AnkG prevented apoptosis with ~65% of AnkG-expressing cells maintaining viability. Ankyrin repeat domains mediate eukaryotic protein–protein interactions (Mosavi et al., 2004) and Dot/Icm substrates predictably bind to, and manipulate, a specific host protein(s). Therefore, Luhrmann et al. (2010) used GST pulldown and immunoprecipitation approaches to identify host-binding partners for AnkG. Interestingly, mass spectrometry analysis and confirmatory immunoprecipitation studies identified the mitochondrial inner matrix protein p32 as a specific AnkG interacting protein. p32 is a pro-apoptotic protein that binds to Hrk to promote cytochrome *c* release (Sunayama et al., 2004) and also interacts with ARF, a pro-apoptotic p53 regulatory protein (Itahana and Zhang, 2008). Thus, AnkG interaction with p32 likely precludes interaction with other pro-apoptotic mitochondrial proteins, resulting in decreased cytochrome *c* release. This is also the first known example of a bacterial protein targeting p32 to manipulate host cell survival.

Next, the authors performed a set of interesting gain of function experiments. In contrast to *Coxiella*, *Legionella* induces rapid apoptosis in some cell types, such as dendritic cells (DCs; Nogueira et al., 2009). Therefore, the authors hypothesized that *Coxiella* AnkG would provide *Legionella* with a tool to inhibit DC apoptosis and allow replication. Mouse bone marrow-derived DCs were infected with *Legionella* expressing *Coxiella* AnkG, then assessed for apoptosis. Remarkably, adding AnkG to *Legionalla*'s effector repertoire reduced DC apoptosis by ~40%. Infecting cells with *Legionella* producing truncated AnkG showed the p32-interacting region was required for inhibition of apoptosis, highlighting the functional importance of an effector binding to a host protein. Additionally, siRNA-mediated dampening of p32 expression reduced *Legionella*-triggered DC apoptosis similar to AnkG production. Collectively, the experiments performed by Luhrmann et al. (2010) provide a mechanism of AnkG anti-apoptotic activity and demonstrate the use one pathogen's effector to study another organism's intracellular activity.

Luhrmann et al. (2010) provide the first glimpse into how a *Coxiella* effector interacts with a host protein to alter a distinct infection event (**Figure 1**). This study also further underscores the differences between *Coxiella* and *Legionella*. Despite similar T4SSs, the effector repertoires of these two pathogens are highly divergent yet required for each organism's intracellular lifestyle. The intriguing results of Luhrmann et al. (2010) also foster some remaining questions about the mechanism of AnkG-mediated protection. First, does translocated AnkG traffic to mitochondria to initiate p32 interactions? AnkG could potentially bind to cytosolic p32, preventing proper localization and interaction with mitochondrial proteins. Second, is AnkG binding to p32 alone sufficient to inhibit cytochrome *c* release? Third, does AnkG influence death receptor-mediated extrinsic apoptosis? Finally, do other Anks contribute to AnkG activity? In their initial screen, the authors showed that AnkF pro-

vides modest protection from apoptosis. Perhaps AnkF works in concert with AnkG to fully protect host cells from death. This prediction is not unprecedented, as four *Legionella* anti-apoptotic effectors target different host proteins (Banga et al., 2007; Ge et al., 2009). Luhrmann et al. (2010) have defined an important role for AnkG in *Coxiella*–host cell interactions and demonstrated elegant experimental approaches that will serve as a blueprint for functional characterization of other *Coxiella* Dot/Icm substrates. It will also be interesting to see if *Legionella* can be exploited to study *Coxiella* effectors that direct other processes such as phagolysosome formation.

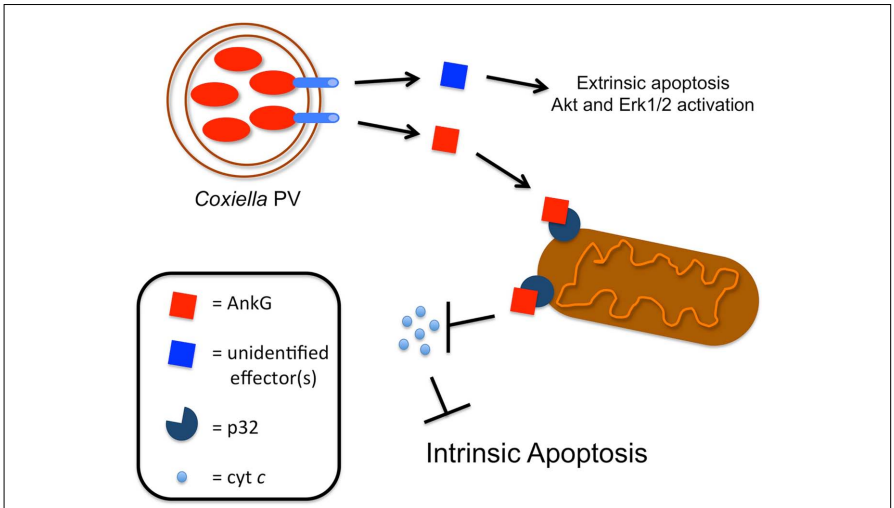

FIGURE 1 | *Coxiella* modulation of apoptosis. *Coxiella* predictably secretes numerous effectors into the host cytosol that influence events including inhibition of extrinsic and intrinsic apoptosis and activation of Akt and Erk1/2. A newly described example is AnkG, which binds specifically to host p32, presumably causing reduced cytochrome *c* (cyt *c*) release, and supplies protection from intrinsic apoptosis.

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Is *C. pneumoniae* research in peril?

Katerina Wolf*

Department of Microbiology and Immunology, University of Miami Miller School of Medicine, Miami, FL, USA
**Correspondence: kwolf@med.miami.edu*

Chlamydia pneumoniae is an obligate intracellular parasite which infects mucosal surfaces of the human respiratory tract causing sinusitis, pharyngitis, bronchitis, and pneumonia. Although the bacterium causes acute disease, mildly symptomatic, asymptomatic, or unrecognized infections are most common (Kuo et al., 1995). *C. pneumoniae* infections are widespread among children 5–14 years of age and by age 20 years about 50% of young adults have detectable antibodies to the microorganism. The seroprevalence to *C. pneumoniae* continues to rise in the population and reaches approximately 75% in the elderly. Moreover, the epidemiological data suggests that most people are infected and re-infected throughout life (Kuo et al., 1995). *C. pneumoniae* was established as a human respiratory pathogen in 1986 (Grayston et al., 1986) and initially research on this pathogen was rigorous, especially due to its association with variety of chronic diseases such as Reiter's syndrome, sarcoidosis, asthma, chronic obstructive pulmonary disease (COPD), multiple sclerosis, Alzheimer disease, and atherosclerosis. However in recent years, the interest in basic as well as clinical research on *C. pneumoniae* has undergone a sharp decline. One of the major factors that could have contributed to this decline may include frequent discrepancies in published data. Multiple studies have described the presence of *C. pneumoniae* in patient samples from those who suffered from the chronic diseases listed above by either serology, polymerase chain reaction (PCR), RT-PCR, immunocytochemistry (ICC) or electron microscopy, and occasionally even by direct isolation of the bacterium. Conversely, there are laboratories which could not confirm these findings. Inter-laboratory variations in sample collection, processing and *C. pneumoniae* detection methods are likely to be responsible for these inconsistencies (reviewed in Boman and Hammerschlag, 2002). However, numerous studies published on *C. pneumoniae*, describing the presence or absence of the microorganism, often lacked appropriate positive and/or negative controls. Using only one or two methods for *C. pneumoniae* detection in clinical specimens is insufficient and may have led to inaccurate conclusions (Puolakkainen et al., 1996; Mills et al., 1998; Sriram et al., 1998; Fainardi et al., 2008). Furthermore, positive labeling of cells or tissue for *C. pneumoniae* antigen(s) with an antibody does not necessarily represent an intact bacterium. It has been demonstrated that chlamydiae-infected cells stay positive for several of the chlamydial antigens, for example LPS, weeks after the bacterium has been degraded by lysosomes (Wolf et al., 2005). Direct isolation of *C. pneumoniae* from a patient's sample still represents the most conclusive, yet the most difficult method of detection of this pathogen. In spite of these inconsistencies there may also be another aspect which could have contributed to the current discrepancies existing in the *C. pneumoniae* field. As mentioned above, the bacterium frequently causes mild or asymptomatic infections, which often remain unrecognized and consequently untreated. In cases such as these, it cannot be conclusively determined at what point during their lifetime the studied patients actually suffered from an acute *C. pneumoniae* infection or re-infection. Many of these concerns could hypothetically be addressed by serology. According to the CDC standards for diagnostic detection of *C. pneumoniae* in clinical samples by micro-immuno-fluorescence (MIF), fourfold rise in IgG titer or an IgM titer ≥16 indicates an acute infection and an IgG titer of ≥16 suggests past exposure to the microorganism. Elevated IgA was excluded as a valid indicator of persistent or chronic infection (Dowell et al., 2001). Unfortunately, the MIF, which is considered the "gold standard" for detection of *C. pneumoniae* in humans does not seem to be absolutely reliable either. Cases of acute, culture-positive *C. pneumoniae* illness without seroconversion have been reported (Kutlin et al., 1998; Hammerschlag and Roblin, 2000). Conversely, the presence of an acute infection detected by MIF has been discovered among subjectively healthy individuals (Hyman et al., 1995). Thus, it is likely that the outcome of *C. pneumoniae* infection depends on the infectious dose and more importantly on fitness of the immune system of each individual. Numerous researchers tend to link various chronic diseases to so called chlamydial persistence. However, very little is known about the actual pathology caused to tissues and/or organs by productive *C. pneumoniae* infection in humans. This considerable lack of basic knowledge concerning the pathogen and its effects on the human body has led many studies to questionable conclusions.

One example of this is represented by reports on secondary prevention of coronary heart disease by treatment of patients who suffered from myocardial infarction with azithromycin (O'Connor et al., 2003; Grayston et al., 2005). In view of the fact that cardiovascular disease is one of the leading causes of fatalities in the developed world, it is not surprising that an association of *C. pneumoniae* with atherosclerosis was a priority to investigate. Participants enrolled in these studies, were largely represented by the older population, who had previously suffered from myocardial infarction and had a *C. pneumoniae* titer of IgG 1:16 or more, indicative of past infection. No data concerning the presence of acute sera was provided (Dunne, 2000; O'Connor et al., 2003; Grayston et al., 2005). The fact that an active *C. pneumoniae* infection was not established in this group of patients is a critical issue. Unfortunately, no validated serological marker for persistent or chronic *C. pneumoniae* infection is currently available (Dowell et al., 2001) and it is highly likely that most of these participants were infected or re-infected with the microorganism in the past, long before they received azithromycin. These studies clearly demonstrated that treatment of patients who had suffered from cardiovascular disease with azithromycin did not reduce or alter the risk

of recurrence of cardiac events. However, they completely failed to address the role, if any, of *C. pneumoniae* in the course of cardiovascular disease (O'Connor et al., 2003; Grayston et al., 2005). An atherosclerotic lesion takes many years to develop during which the patient could have encountered asymptomatic *C. pneumoniae* infection(s) at any time. If the bacterium does contribute to various chronic diseases one must not disregard the possibility that an injury to the arteries and other parts of our body may be achieved during the course of an acute respiratory infection with *C. pneumoniae*. If the microorganism is ever shown to cause or worsen any of the proposed chronic diseases, research on this pathogen must first properly investigate primary infection within the human respiratory tract. Similarly to *C. trachomatis*, infections with *C. pneumoniae* are often asymptomatic meaning that the infected person is unlikely to receive proper antibiotic treatment. Therefore clearance of the pathogen must entirely depend on the immune response of the host. It still remains unclear whether any of the diseases, respiratory or non-respiratory, linked to *C. pneumoniae* are caused directly by chlamydial growth or by the immune system's attempt to resolve the infection or both. The recent body of evidence suggests that *C. pneumoniae* employs sophisticated, species-specific strategies, which are comparatively more efficient than those utilized by *C. trachomatis*, in order to avoid recognition by the host innate immune system (Wolf et al., 2009 and unpublished data).

Although all chlamydiae share similarities in biology, they also display extraordinary diversity in tissue tropism and disease manifestation. Based on comparisons among sequenced chlamydial genomes, it seems that *C. pneumoniae* represents an intriguing chlamydial species with unique features, all of which are worthy of further investigation. For example, the *C. pneumoniae* chromosome contains a plasticity zone, a region with a higher rate of DNA reorganization, which is three times larger than the plasticity zone of *C. trachomatis* (~160 versus ~50 kb, respectively; Read et al., 2000). It is postulated that the gene content within the plasticity zone significantly contributes to the virulence of each chlamydial species. Moreover, *C. pneumoniae* contains 21 *pmp* genes encoding chlamydial polymorphic membrane proteins compared to only nine detected in *C. trachomatis*. Overall, the *C. pneumoniae* genome is ~0.15 Mb larger than that of *C. trachomatis* and contains 214 coding sequences which are not found in *C. trachomatis* (Kalman et al., 1999). *C. pneumoniae* is an established pathogen causing a significant number of respiratory infections in humans. However, at this point there is insufficient data with regard to the basic biology of this microorganism that would help to elucidate pathogenic strategies employed by *C. pneumoniae* in order to achieve successful invasion of its human host. In conclusion, more rigorous and thorough research on *C. pneumoniae* is absolutely essential for better understanding of its virulence within the human respiratory tract and its potential association with various chronic diseases.

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Global proteomic analysis of two tick-borne emerging zoonotic agents: *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*

Mingqun Lin[1], Takane Kikuchi[1], Heather M. Brewer[2], Angela D. Norbeck[2] and Yasuko Rikihisa[1]*

[1] *Department of Veterinary Biosciences, The Ohio State University, Columbus, OH, USA*
[2] *Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, WA, USA*

Edited by:
Robert Heinzen, NIH/NIAID-RML, USA

Reviewed by:
Michael F. Minnick, University of Montana, USA
Jere W. McBride, University of Texas Medical Branch, USA
Sanjeev K. Sahni, University of Rochester School of Medicine and Dentistry, USA

***Correspondence:**
Yasuko Rikihisa, Department of Veterinary Biosciences, The Ohio State University, 1925 Coffey Road, Columbus, OH 43210, USA.
e-mail: rikihisa.1@osu.edu

Anaplasma phagocytophilum and *Ehrlichia chaffeensis* are obligatory intracellular α-proteobacteria that infect human leukocytes and cause potentially fatal emerging zoonoses. In the present study, we determined global protein expression profiles of these bacteria cultured in the human promyelocytic leukemia cell line, HL-60. Mass spectrometric (MS) analyses identified a total of 1,212 *A. phagocytophilum* and 1,021 *E. chaffeensis* proteins, representing 89.3 and 92.3% of the predicted bacterial proteomes, respectively. Nearly all bacterial proteins (≥99%) with known functions were expressed, whereas only approximately 80% of "hypothetical" proteins were detected in infected human cells. Quantitative MS/MS analyses indicated that highly expressed proteins in both bacteria included chaperones, enzymes involved in biosynthesis and metabolism, and outer membrane proteins, such as *A. phagocytophilum* P44 and *E. chaffeensis* P28/OMP-1. Among 113 *A. phagocytophilum p44* paralogous genes, 110 of them were expressed and 88 of them were encoded by pseudogenes. In addition, bacterial infection of HL-60 cells up-regulated the expression of human proteins involved mostly in cytoskeleton components, vesicular trafficking, cell signaling, and energy metabolism, but down-regulated some pattern recognition receptors involved in innate immunity. Our proteomics data represent a comprehensive analysis of *A. phagocytophilum* and *E. chaffeensis* proteomes, and provide a quantitative view of human host protein expression profiles regulated by bacterial infection. The availability of these proteomic data will provide new insights into biology and pathogenesis of these obligatory intracellular pathogens.

Keywords: *Anaplasma phagocytophilum, Ehrlichia chaffeensis*, proteomic analysis, human granulocytic anaplasmosis, human monocytic ehrlichiosis, human leukocytes

INTRODUCTION

Anaplasma phagocytophilum and *Ehrlichia chaffeensis* are small (ca. 0.4 by 1.5 μm), pleomorphic gram-negative bacteria that belong to the family Anaplasmataceae in the order Rickettsiales, the class α-proteobacteria (Dumler et al., 2001; Rikihisa, 2010b). The infection of humans by *A. phagocytophilum* and *E. chaffeensis* causes human granulocytic anaplasmosis [HGA, first reported in 1994, formerly known as human granulocytic ehrlichiosis (HGE)] and human monocytic ehrlichiosis (HME, first reported in 1987), respectively (Maeda et al., 1987; Chen et al., 1994). HGA and HME are similar systemic febrile diseases characterized by fever, headache, myalgia, anorexia, and chills, and are frequently accompanied by leukopenia, thrombocytopenia, anemia, and elevations in serum hepatic aminotransferases (Paddock and Childs, 2003; Bakken and Dumler, 2008; Thomas et al., 2009). Neurological signs are more frequently reported with HME than HGA (Paddock and Childs, 2003). Although doxycycline is generally effective in treating human ehrlichioses, delayed therapy, the presence of underlying allergies or poor health, and immuno-suppression often lead to severe complications or death. As important life-threatening tick-borne emerging zoonoses, HGA and HME were designated as nationally notifiable diseases by US Centers for Disease Control and Prevention in 1998 (Gardner et al., 2003). Since them, reported cases have increased every year. During 2008, cases attributed to *A. phagocytophilum* and *E. chaffeensis* increased by 21 and 16% from 2007, respectively (Hall-Baker et al., 2010).

Anaplasma phagocytophilum and *E. chaffeensis* are obligatory intracellular bacteria with a life cycle that requires repeated transmission between mammalian hosts and tick vectors (Rikihisa, 1991, 2010b; Dumler et al., 2001). Once transmitted to mammals, these bacteria replicate in membrane-bound compartments inside the primary host immune defensive cells: granulocytes (*A. phagocytophilum*) or monocytes/macrophages (*E. chaffeensis*). Since culture isolation of these organisms in the 1990s (Dawson et al., 1991; Goodman et al., 1996), unique strategies employed by *A. phagocytophilum* and *E. chaffeensis* for their survival in hostile environment have begun to be unraveled, including hijacking host cell signaling pathways, altering vesicular trafficking, usurping nutritional and cytoskeletal components, and subverting several host innate immune responses (Carlyon and Fikrig, 2003, 2006; Carlyon et al., 2004; Sukumaran et al., 2005; Huang et al., 2010a; Rikihisa, 2010a,b; Sultana et al., 2010; Wakeel et al., 2010). The complete genome sequences of *A. phagocytophilum* (1,471,282 base pairs) and *E. chaffeensis* (1,175,764 bp), and detailed analyses of their protein-coding genes have proven a

great resource for studying these bacteria and the diseases they cause (Dunning Hotopp et al., 2006). These two species share approximately 500 genes; most of them encode proteins with homologies to those with known functions. However, approximately 470–580 genes are unique to each species (Dunning Hotopp et al., 2006), and approximately 45% of predicted open reading frames (ORFs) in the two genomes were annotated as uncharacterized "hypothetical proteins" or proteins without any functional assignment (**Table 1**). However, whether they really encode proteins and are actually expressed in living organisms remains largely unknown.

Owing to the recent technical advance in transcriptome and proteome analyses, a holistic view of the numerous expressed genes and proteins of an organism has become available. Whole genome transcriptome analysis of *A. phagocytophilum* in human HL-60 cells showed the expression of approximately 70% of the bacterial gene transcripts (Nelson et al., 2008). Proteomics studies based on 1-D and 2-D gel analyses of *E. chaffeensis* identified one-fourth of the total ORFs from human and tick cell-derived bacterial cultures (Singu et al., 2005; Seo et al., 2008). However, there are major difficulties in proteomic studies of obligatory intracellular bacteria; because a high-purity bacterial sample is not easily obtainable, and the presence of a large amount of host proteins reduces the sensitivity and lowers the identification scores of bacterial proteins (Li and Lostumbo, 2010). The development of more sensitive nano-liquid chromatography combined with tandem MS/MS (nano-LC–MS/MS)-based proteomic approach improves global protein analysis of obligatory intracellular bacteria, as low levels of proteins can be identified in samples mixed with a large amount of host proteins (Zimmer et al., 2006). Furthermore, label-free protein quantitation based on LC–MS peptide peak intensity information becomes possible due to the reproducibility and sensitivity of intensity data measurements, and multiple samples from different conditions can be compared directly without stable isotope labeling (Old et al., 2005; Zimmer et al., 2006; Shi et al., 2009).

Here, we present the first comprehensive proteomes of two human pathogens *A. phagocytophilum* and *E. chaffeensis*, their relative protein expression abundances, and the influence of infection with these two pathogens on human host protein expression using multidimensional nano-LC–MS/MS approaches developed at Pacific Northwest National Laboratory[1] (Zimmer et al.,

[1]http://omics.pnl.gov/

Table 1 | Numbers of protein expression classified by functional categories.

	Anaplasma phagocytophilum			*Ehrlichia chaffeensis*		
Functional categories[1]	**Total**	**Expressed**	**Not detected**	**Total**	**Expressed**	**Not detected**
Amino acid biosynthesis	9	9		23	23	
Biosynthesis of cofactors, prosthetic groups, and carriers	64	64		61	61	
Cell envelope	159	156	3	49	49	
Cellular processes	29	29		29	29	
Central intermediary metabolism	2	2		3	3	
DNA metabolism	46	46		43	43	
Energy metabolism	87	87		83	83	
Fatty acid and phospholipid metabolism	18	18		19	19	
Mobile and extrachromosomal elements	6	6		4	4	
Protein fate	82	79	3	78	77	1
Protein synthesis	106	105	1	106	106	
Purines, pyrimidines, nucleosides, and nucleotides	36	36		35	35	
Regulatory functions	9	9		11	11	
Transcription	21	21		20	20	
Transport and binding proteins	34	34		31	30	1
Proteins with known functions	**708**	**701 (99.0%)**	**7**	**595**	**593 (99.7%)**	**2**
Proteins <100 AA	55	49 (89.1%)		34	32 (94.1%)	
Proteins with unknown functions[2]	**610**	**475 (77.9%)**	**135**	**504**	**422 (83.7%)**	**82**
"Hypothetical" proteins <100 AA	367	234 (63.8%)		243	161 (66.3%)	
Truncated ORFs[3]	39	36	3	7	6	1
Summary	**1357**	**1212 (89.3%)**	**145**	**1106**	**1021 (92.3%)**	**85**
Disrupted ORFs (no AA translation)[4]	13	0	13	10	0	10
Total ORF numbers	**1370**	**1212**	**158**	**1116**	**1021**	**95**

[1]Function categories are assigned by the JCVI Annotation Engine and available at JCVI Comprehensive Microbial Resource (http://cmr.jcvi.org).
[2]ORFs of unknown functions refer to hypothetical proteins and proteins without functions assigned.
[3]Truncated ORFs refer to truncated or frame-shifted proteins of known, characterized ORFs in the NCBI database.
[4]Disrupted ORFs refer to ORFs (except p44s in A. phagocytophilum) that have homologs to annotated ORFs in GenBank but contain nonsense mutations.

2006; Mottaz-Brewer et al., 2008). The determination of protein expression profiles of *A. phagocytophilum* and *E. chaffeensis* in human leukocytes will help advance understanding cell biology, physiology of these bacteria, and complex interplay between bacteria and their host, and enhance the opportunities for investigation of novel targets for antimicrobial therapy or blocking of pathogenic pathways.

MATERIALS AND METHODS

BACTERIA CULTURE AND PURIFICATION

Anaplasma phagocytophilum HZ (type strain; Rikihisa et al., 1997) and *E. chaffeensis* Arkansas (type strain; Dawson et al., 1991) were cultured in HL-60 cells, which are undifferentiated human promyelocytic leukemia cells from ATCC (#CCL-240, Manassas, VA, USA). Cells were maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum and 2 mM L-glutamine, and incubated at 37°C in a humidified 5% CO2–95% air atmosphere. No antibiotic was used throughout the study. When infectivity reached greater than 95% as assessed by Diff-Quik staining of cytocentrifuged preparations (Baxter Scientific Products, Obetz, OH, USA), infected cells were harvested, extensively washed to remove serum proteins, and host cell-free bacteria were released by sonication for 10 s at an output setting of 2 with an ultrasonic processor W-380 (Heat Systems, Farmington, NY, USA). After low-speed centrifugation at 700×*g* to remove nuclei and unbroken cells, the supernatant was filtered through a 5-μm then 0.8-μm filter (Millipore, Billerica, MA, USA) to remove cellular debris. The filtrate was then centrifuged at 10,000 × *g* for 10 min, and the pellet enriched with host cell-free bacteria was collected.

SAMPLE PREPARATION FOR PROTEOMICS ANALYSIS: PROTEIN PARTITIONING, DIGESTION, AND CLEAN-UP

To obtain comprehensive coverage of protein expression profiles, including both hydrophilic and hydrophobic proteins, proteins with very high or low pIs, and proteins with different cellular distributions, three optimized protein extraction protocols, including global, soluble, and insoluble protein extracts, were applied to purified host cell-free bacteria and uninfected or infected HL-60 cells as described previously (Mottaz-Brewer et al., 2008). For tryptic digestion with global protein extracts, pellets containing purified bacteria or host cells were suspended in 100 mM NH_4HCO_3 buffer (pH 8.4). The resulting suspension was transferred to a 2.0-mL cryovial tube with O-ring in cap, and lysed by beating with 0.1-mm zirconia/silica disruption beads (BioSpec Products, Bartlesville, OK, USA). Protein samples were denatured and reduced by adding urea, thiourea, and dithiothreitol (DTT) at final concentrations of 7 M, 2 M, and 5 mM, respectively. Following incubation at 60°C for 30 min, the samples were diluted 10-fold with NH_4HCO_3 buffer. Global digest was performed by adding trypsin at 1:50 (w:w) enzyme:protein ratio, and $CaCl_2$ at a final concentration of 1 mM. The samples were incubated at 37°C for 3 h, snap frozen in liquid N_2 to stop the digestion, and stored at −80°C until further analysis. Clean-up was performed using a Discovery C-18 solid phase extraction (SPE) column (Supelco, Bellefonte, PA, USA) to prepare the samples for MS analysis. Peptides were then concentrated by a Savant SpeedVac manifold (Thermo Fisher, Milford, MA, USA), and a BCA protein assay (Thermo Fisher/Pierce, Rockford, IL, USA) was performed to determine the final sample concentration.

For digestion with soluble and insoluble protein extracts, purified bacterial or host cell pellets were resuspended in 50 mM NH_4HCO_3 buffer and centrifuged at 355,000×*g* at 4°C for 10 min to separate the protein lysates into two parts: soluble and insoluble protein fractions. The supernatant was tryptically digested and cleaned up in the same fashion as in the global digest method and designated as soluble digest samples. The pellet after ultracentrifugation, containing the insoluble protein fraction, was washed and resuspended in a denaturing solution (7 M urea, 2 M thiourea, 1% CHAPS, 10 mM DTT, 50 mM NH_4HCO_3, pH 7.8). Insoluble protein samples were digested as described above. Removal of salts and detergent was performed using a Discovery strong cation exchange (SCX) SPE column (Supelco). Peptides were concentrated, and the concentration measured as described above. All trypsin-digested peptides were snap frozen in liquid N_2 and stored at −80°C until proteomic analysis.

MASS SPECTROMETRY AND DATA ANALYSIS

In order to enhance proteome coverage, all peptide samples were further separated by SCX chromatography coupled off-line with nano-LC–MS/MS analyses. Peptide mixtures from each proteome sample were fractionated into 35–70 fractions as previously described (Qian et al., 2005). A description of the instrumentation and specifics of the high-performance liquid chromatography (HPLC–MS/MS) and HPLC–MS instrumental arrangements and associated methods for each biological system have been described previously and are consistent for all experiments (Manes et al., 2008; Mottaz-Brewer et al., 2008). In brief, samples were loaded onto an in-house developed chromatography system that uses a 20-cm × 75-μm C18 reverse-phase column and ionized as they eluted from the column into a mass spectrometer using electrospray ionization. The liquid chromatography gradient was generated linearly from aqueous to organic over 100 min in acidic conditions. Typically, MS was performed in a linear trap quadrupole (LTQ; Thermo Fisher Scientific) ion trap mass spectrometer. Tandem MS (MS/MS) were collected using data-dependent settings on the top 10 ions from the precursor scan.

Tandem MS spectra (MS/MS) were matched to protein sequence files using the SEQUEST program and filtered with a combination of scores provided in the output files (Eng et al., 1994), which included the minimum threshold filter scores defined by Washburn et al. (2001), and an additional minimum discriminant score of 0.5 to reduce the false-positive identifications (Strittmatter et al., 2004). Only peptides passing these filters were populated into the initial accurate mass and time (AMT) tag database. The searches were performed using the annotated protein databases of *A. phagocytophilum* HZ (1,357 protein entries, GenBank Accession Number NC_007797) including newly annotated 113 *A. phagocytophilum* P44 proteins, *E. chaffeensis* Arkansas (1,106 protein entries, GenBank Accession Number NC_007799), and *Homo sapiens* IPI protein database (61,225 protein entries, IPI 2006, v3.36). Each bacterial and human peptide from infected host cells was identified and populated into the same AMT tag database.

QUANTITATIVE MASS SPECTROMETRIC ANALYSIS

Before running on the mass spectrometer, the total peptide mass was measured, and the sample was diluted to 1 μg/μL for injection. After the building of the initial AMT tag database, all samples were analyzed with a 9.4-T Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (Bruker Daltonic, Billerica, MA, USA) following the separation of peptides by reverse-phase capillary HPLC under identical conditions as described (Shi et al., 2006, 2009). Standard proteins were added before digestion and used to track the performance of the instruments. Five technical replicates for each sample were injected into the FTICR mass spectrometer. Relevant information, such as the elution time from the capillary LC column, the abundance of the signal (integrated area under the elution profile), and the monoisotopic mass (determined from charge state and the high accuracy *m*/*z* measurement) of each feature observed in the FTICR, was used to match the peptide identifications contained within the initial AMT tag database. These peptides, now identified and quantified, were used to infer the protein composition of the samples. Only peptides observed in at least three out of five of these technical replicates were used in data analysis, and all proteins were required to have at least three observed peptides to be included in the confident results. In addition, the number of peptides observed for each protein in a biological sample was divided by the total number of peptides determined from the same sample to give an estimate of relative abundance of each identified protein in the sample. The abundances for each peptide were then averaged across these runs before the ratio calculations.

DATA ANALYSIS AND PROTEOMIC DATABASE ONLINE ACCESS

Bacterial proteins were classified based on the functional role categories using JCVI Annotation Engine and Comprehensive Microbial Resource[2] as described previously (Dunning Hotopp et al., 2006; Lin et al., 2009). Human proteins were classified based on gene ontology (GO) as annotated by the GO Consortium[3]. All peptides and proteins identified from *A. phagocytophilum*, *E. chaffeensis*, and human HL-60 cells, together with the detailed analyses of protein expression profiles and quantitation results can be accessed and downloaded from the website[4].

RESULTS AND DISCUSSION

OVERVIEW OF *A. PHAGOCYTOPHILUM* AND *E. CHAFFEENSIS* PROTEINS IDENTIFIED BY PROTEOMICS

In order to identify and quantitate the comprehensive protein expression profiles of *A. phagocytophilum* and *E. chaffeensis*, 18 *A. phagocytophilum* protein samples and 14 *E. chaffeensis* samples were prepared from purified host cell-free bacteria and infected HL-60 cells using three different protein extraction protocols as described; each contained approximately 1 mg of peptides after tryptic digestion and column clean-up. Approximately 250 MS runs for each bacterium from these samples were performed until no new peptides were detected (**Figure A1** in Appendix). Positively identified peptides were populated into the AMT tag database, and two databases were constructed. The *A. phagocytophilum* database contained search results from 60 datasets using purified bacteria from infected cells and 189 datasets from *A. phagocytophilum*-infected cells, respectively. The database for *E. chaffeensis* contained 49 datasets from purified bacteria and 192 datasets associated with *E. chaffeensis*-infected host cells.

In protein samples from both *A. phagocytophilum* and *E. chaffeensis*-infected HL-60 cells, greater than 126,000 peptides were identified (**Figure A1** in Appendix). Among these peptides, 44,080 matched to 1,212 *A. phagocytophilum* proteins, and 40,004 matched to 1,021 *E. chaffeensis* proteins, representing 89.3 and 92.3% of the predicted bacterial proteomes, respectively (**Table 1**). Among these detected proteins, greater than 96% have more than one peptide match. For proteins with known functional categories assigned, nearly all of these proteins (99.0% from *A. phagocytophilum* and 99.7% from *E. chaffeensis*) were expressed in HL-60 cells, including enzymes required for metabolisms and proteins involved in pathogenesis and regulatory functions, such as outer membrane proteins, the type IV secretion system (T4SS), and two-component regulatory systems. Therefore, nearly all proteins with known functions are likely essential for the replication and survival of these two pathogens inside human host cells. These expression profiles in mammalian host also suggest that, although gene loss occurred in the family Anaplasmataceae as a result of reductive genome evolution (Blanc et al., 2007; Darby et al., 2007), these genes cannot be sacrificed from their genomes.

Among these currently identified bacterial proteins, some mRNAs or proteins have been reported previously, including 70% of *A. phagocytophilum* genes in HL-60 cells by whole genome transcriptome analysis (Nelson et al., 2008) and near one-fourth of *E. chaffeensis* proteins by 1-D and 2-D gel based proteomics studies (Singu et al., 2005; Seo et al., 2008). Dozens of surface-exposed proteins in both bacteria using NHS-SS-biotin-labeling of host cell-free, intact bacteria cultured in human host cells (Ge and Rikihisa, 2007a,b). All of these identified proteins were detected in the current proteomic analyses. In addition, several other bacterial proteins reported previously were also detected in this study. These proteins include outer membrane proteins P44 and P28/Omp-1 (Ohashi et al., 1998b; Unver et al., 2002; Zhi et al., 2002; Zhang et al., 2004; Huang et al., 2007; Kumagai et al., 2008); transcriptional factors Tr1, ApxR, and EcxR (Wang et al., 2007; Cheng et al., 2008); three pairs of two-component regulatory system proteins in both bacteria (Cheng et al., 2006; Kumagai et al., 2006); the VirB/D4 T4S apparatus and substrates like AnkA and Ats-1 (Caturegli et al., 2000; Ohashi et al., 2002; IJdo et al., 2007; Lin et al., 2007; Bao et al., 2009; Niu et al., 2010); *A. phagocytophilum* toxin A (AptA) protein and morulae proteins APH_0032/APH_1387 (Huang et al., 2010b,c; Sukumaran et al., 2011); and *E. chaffeensis* Ank200 and tandem repeat proteins Trp32/Trp47/Trp120 (Wakeel et al., 2009, 2010; Zhu et al., 2009; Luo et al., 2010).

EXPRESSION OF *A. PHAGOCYTOPHILUM* AND *E. CHAFFEENSIS* PROTEINS IN BIOSYNTHESIS PATHWAYS AND PHAGE COMPONENTS

Anaplasma phagocytophilum and *E. chaffeensis* have significantly higher percentages of their genomes involved in nucleotide biosynthesis, cofactor and vitamin biosynthesis, and protein synthesis than their closely related free-living α-proteobacterium *Caulobacter crescentus* (Dunning Hotopp et al., 2006). Expression of enzymes

[2]http://cmr.jcvi.org

[3]http://www.geneontology.org/

[4]http://riki-lb1.vet.ohio-state.edu/proteomics/

involved in nucleotide, vitamin, and cofactor biosynthetic pathway in *A. phagocytophilum* and *E. chaffeensis*, suggests that they do not need to compete with human leukocytes for, and may even supply host cells with, essential vitamins and nucleotides. This has been proposed to occur between the obligatory intracellular bacterium *Wigglesworthia glossinidia* and its insect host, tsetse fly (Zientz et al., 2004). The protein synthesis category includes many essential genes, such as those encoding ribosomal proteins, tRNA synthetases, RNA modification enzymes, and translation factors. Almost all of these proteins were expressed in mammalian hosts, except for *A. phagocytophilum* ribosomal protein L36 (**Table S1** in Supplementary Material). Previous studies have shown that ribosomal protein L36 is dispensable for *Escherichia coli* growth and protein synthesis (Ikegami et al., 2005), and the gene encoding ribosomal protein L36 was not identified in the closely related *Neorickettsia* spp (Lin et al., 2009), suggesting that L36 might not be necessary for members in the family Anaplasmataceae.

Anaplasma phagocytophilum and *E. chaffeensis* have a lower coding percentage for transporters compared to the free-living *C. crescentus* (Dunning Hotopp et al., 2006). Although nearly 100% of the proteins with known functions were expressed in HL-60 cells, few proteins involved in transport functions, like twin-arginine translocation protein TatA/E of *A. phagocytophilum*, and monovalent cation/proton antiporter MnhG/PhaG subunit family protein of *E. chaffeensis* were not detected in bacteria cultured in HL-60 cells (**Tables S1 and S2** in Supplementary Material). Interestingly, although *A. phagocytophilum* and *E. chaffeensis* do not encode for intact prophage or transposable/mobile elements, a few phage core components (HK97-like portal, major capsid, and prohead protease) were identified scattered throughout their genomes, and their protein expressions were also confirmed by proteomics. The functions of these remnant phage components on bacterial infection of human hosts are unclear; however, some literature has suggested that they might be involved in lateral gene transfer, bacterial chromosome inversion, evolution, and virulence factors expression (Canchaya et al., 2003; Brussow et al., 2004).

EXPRESSION PROFILING OF *A. PHAGOCYTOPHILUM* AND *E. CHAFFEENSIS* "HYPOTHETICAL" PROTEINS

Since approximately 45% of the predicted ORFs in the genomes encode conserved or uncharacterized "hypothetical" proteins (**Table 1**; Dunning Hotopp et al., 2006), whether they really encode proteins and whether these proteins are expressed in living organisms are largely unknown. Analysis of the expression profiles of these hypothetical proteins or proteins without known functions assigned showed that only 77.9 and 83.7% of them were expressed in *A. phagocytophilum* and *E. chaffeensis*, respectively (**Table 1**). The much lower expression ratio of these "hypothetical" genes compared to those of proteins with known functions assigned (near 100%), suggests that the expression of certain "hypothetical" proteins might be regulated in different host environments, like the arthropod vectors, and play critical roles in responses to host adaptation.

As suggested by Ochman (2002) and Skovgaard et al. (2001), a substantial fraction of hypothetical ORFs in bacterial genomes are short (under 300 nucleotides in length); therefore, many of them might be random stretches of DNA and do not actually encode proteins. Analysis of these expressed proteins with unknown functions showed that 50.7% of them in *A. phagocytophilum* and 61.8% in *E. chaffeensis* were greater than 100 amino acids (AA) in protein length (**Tables S3 and S4** in Supplementary Material). However, for "hypothetical" proteins undetectable by proteomic analysis, 97.8% of them in *A. phagocytophilum* and 100% in *E. chaffeensis* were fewer than 100 AA (**Table 2**). As functional assignment to an ORF during genome annotation process is based on the homology or domain structure matches to known proteins or domains, proteins with known functions assigned are most likely biased toward long proteins (Skovgaard et al., 2001). This statement is probably true since among the "hypothetical" proteins, 60.2% in *A. phagocytophilum* and 48.2% in *E. chaffeensis* are fewer than 100 AA, whereas less than 8% of proteins with known functions are fewer than 100 AA (**Table 1**). The shorter protein length also reduces its possibility of being detected by proteomic analysis due to the smaller number of peptides after trypsin-treatment. However, our study showed that greater than 63% of the "hypothetical" proteins with fewer than 100 AA could be detected in both bacteria (**Table 1**). Therefore, further bioinformatics analyses of these expressed genes, combined with comprehensive protein expression profiles under different culturing or host environmental conditions, would help in the prediction of true "hypothetical" proteins.

EXPRESSION OF OVERLAPPING ORFS IN *A. PHAGOCYTOPHILUM* AND *E. CHAFFEENSIS*

Overlapping genes are detected primarily in parasitic or symbiotic bacteria and are believed to be a consequence of the reduction of originally larger genomes (Fukuda et al., 2003; Blanc et al., 2007). Analyses of the *A. phagocytophilum* and *E. chaffeensis* genomes identified overlaps among protein-coding ORFs and between RNA- and protein-coding ORFs, which occurred either at different reading frames of the same strand or on the complementary strands. Proteomic data showed that many overlapping genes were indeed expressed by *A. phagocytophilum* and *E. chaffeensis* in infected human host cells (**Figures A2 and A3** in

Table 2 | Classification of undetected proteins by functional categories and protein lengths.

Organisms	*Anaplasma phagocytophilum*		*Ehrlichia chaffeensis*	
Proteins with assigned functions	7		2	
Protein length <100 AA		6		2
Proteins with unknown functions	135		82	
Protein length <100 AA		132 (97.8%)		82 (100%)
Truncated ORFs	3		1	
Protein length <100 AA		3		1
Total numbers of undetected ORFs	**145**		**85**	
Numbers of protein <100 AA		**128 (97.2%)**		**85 (100%)**
Disrupted ORFs (no translation)	**13**		**10**	

Appendix). These ORFs include one pair each of completely overlapping protein-coding ORFs in *A. phagocytophilum* (APH_0143/APH_0144) and *E. chaffeensis* (ECH_0506/ECH_0507), one pair of overlapping ORFs between protein (ECH_0472) and 6SRNA1 genes (ECH_1158), and 10 out of 21 (*A. phagocytophilum*) or 4 out of 26 (*E. chaffeensis*) partial overlapping protein-coding ORFs (**Figures A2 and A3** in Appendix). These data suggest that overlapping ORFs can actually be transcribed and translated into proteins in these organisms with reduced genome contents in order to increase their coding capacities.

EXPRESSION OF P44/MSP2 AND OMP-1 PROTEIN SUPERFAMILY IN *A. PHAGOCYTOPHILUM* AND *E. CHAFFEENSIS*

Despite the reduction in their genome sizes and significantly lower coding capacity for metabolism, transport, and regulatory functions, *A. phagocytophilum* and *E. chaffeensis* not only retained but expanded a pool of genes encoding outer membrane proteins (Dunning Hotopp et al., 2006). Most of these outer membrane proteins are members of Pfam PF01617 and constitute the OMP-1/MSP2/P44 family (Dunning Hotopp et al., 2006; Finn et al., 2010). Since *A. phagocytophilum* and *E. chaffeensis* cannot be transovarially transmitted in their arthropod vectors, and ticks must acquire these organisms by feeding on an infected vertebrate reservoir animal, it was proposed that the expansion of this gene family might allow persistence in the vertebrate reservoir by providing antigenic variation, thus allowing for effective transmission from mammals to ticks (Rikihisa, 2010a).

The *A. phagocytophilum* genome has the largest expansion of the genes belonging to OMP-1/MSP2/P44 family among members of the family Anaplasmataceae, most of them encoding P44 outer membrane proteins (Dunning Hotopp et al., 2006). A total of 113 annotated *p44* loci longer than 60 bp in gene length and some smaller DNA fragments homologous to *p44* gene family can be identified throughout the genome, which consists of greater than 5% of the total genome contents (Dunning Hotopp et al., 2006). The full-length *p44*s consist of a central hypervariable region of approximately 280 bp encoding a signature of four conserved AA regions (C, C, WP, A) and conserved flanking sequences (**Table 3**; Lin and Rikihisa, 2005; Dunning Hotopp et al., 2006). By comparing the length and domains of the identified *p44*s to the full-length *p44*s, all *p44* genes were annotated and classified as full-length, truncated, fragmented, or degenerated genes, as defined in **Table 3** (Dunning Hotopp et al., 2006). Due to the lack of start/stop codons, silent *p44* gene fragments are unlikely to be expressed at their own genomic loci and have to be recombined into and expressed from the expression locus APH_1221 (*p44-18ES*) by a RecF-dependent recombination, as suggested by Lin et al. (2006). To assist proteomic detection of all possible P44 peptides, in-frame AA sequences were deduced from all *p44* genes (including silent pseudogenes without start/stop codons and degenerated *p44* fragments containing nonsense mutations) and used in the SEQUEST search. The expression of P44-59 in the *A. phagocytophilum* outer membrane from these pseudogenes had been confirmed previously (Ge and Rikihisa, 2007a). Results showed that in addition to 22 full-length P44s, peptides identified by proteomic analysis were matched to protein sequences deduced from nearly all *p44* genes (97.3%), including 86 silent *p44* gene fragments and 2 degenerated *p44* genes (**Table 3**).

Table 3 | Expression profile of *Anaplasma phagocytophilum* P44 outer membrane proteins.

P44 proteins[1]	Total numbers detected	All P44 with peptides peptides detected	Expressed P44 w/unique
Full-length P44 proteins	22	22	19[2]
Truncated P44 fragments	68	67[3]	52
N- or C-terminal P44 fragments	21	19[4]	11
Degenerated P44 fragments	2	2	2
Total numbers	**113**	**110 (97.3%)**	**84 (74.3%)**

[1]Full-length p44 genes are longer than 1,000 bp, contain conserved start and stop codons, encode both of the conserved N- and C-regions and a central hypervariable region. These genes can be expressed at their respective current genomic loci or can recombine into the expression locus p44-18ES (APH_1221).
Truncated p44s are silent/reserve p44s less than 1,000 bp in length, encode the complete or a portion of the central hypervariable region, and have one or both of the conserved N- and C-regions. They may have alternative start and/or stop codons.
Fragments of p44 have only a conserved region and no hypervariable region and are longer than 60 bp. It should be noted that smaller fragments can be identified throughout the genome.
Degenerated p44 fragments are p44 truncations that are likely to be non-functional remnants of previous recombination events and contain nonsense mutations.
[2]Three P44s have peptides detected, but all of these peptides also matched to other P44s: P44-2b (shared with P44-2), P44-34b (shared with P44-34), P44-53b (shared with P44-53).
[3]Only P44-75 protein (APH_1122) has no peptide matches.
[4]Two P44 C-terminal fragments APH_1124 and APH_1399 were not detected.

Since the N- and C-regions flanking hypervariable domains are highly conserved among P44 proteins, one peptide identified by proteomic analysis might actually match to several P44s. Therefore, we further analyzed all peptide matches to P44 proteins and confirmed that 84 P44 proteins (74.3%) were expressed with at least one unique peptide match (**Table 3**; **Table S5** in Supplementary Material). These results showed that silent *p44* gene reserves distributed throughout the *A. phagocytophilum* genome can actually be recombined and expressed from the *p44-18ES* expression locus (**Figure 1**). In addition, the region near this expression locus showed greater numbers of identified peptides matched to P44 proteins encoded by either full-length *p44* genes that can be expressed at their own loci or silent *p44* genes that have to be recombined into the *p44-18ES* locus for protein expression (**Figure 1**), which could be due to higher transcription activities in this region and/or higher recombination activities with the *p44-18ES* locus. The expression of the entire P44 repertoire by populations of this bacterium in human leukocytes would ensure their rapid adaptation to changing host environments and successful parasitism in new host cells, as well as escaping host immune surveillance. These results confirm our previous findings from mRNA data that diverse P44s can be expressed at the *p44*-expression locus by gene conversion from over 100 *p44* donor loci (Lin et al., 2003, 2004; Wang et al., 2004; Lin and Rikihisa, 2005).

Ehrlichia chaffeensis has 22 paralogous tandemly arranged *p28/omp-1* genes encoding immunodominant major outer membrane proteins (Ohashi et al., 1998a,b; Dunning Hotopp et al., 2006). Proteomics analyses showed that all these proteins and 27 other cell envelope proteins are expressed by *E. chaffeensis* in HL-60 cells (**Table 1**). Nineteen out of 22 P28/OMP-1 proteins have also been confirmed by proteomic identification of surface-exposed proteins of *E. chaffeensis* cultured in the human acute leukemia cell line THP-1 (Ge and Rikihisa, 2007b). Temporal transcript analyses showed that mRNA expression of 16 out of 22 *p28/omp-1* genes was detected in the blood from acute to chronically infected dogs (over 56 days of infection; Unver et al., 2002). Using 22 synthetic antigenic peptides unique to each of the P28/OMP-1 proteins, sera from persistently infected dogs were reacted with all P28/OMP-1 family proteins (Zhang et al., 2004). These data suggest that P28/OMP-1 family proteins are not involved in immune evasion at the population level (Unver et al., 2002; Zhang et al., 2004).

Surface expression of porins that function as passive diffusion channels is required for small hydrophilic compounds to pass through the outer membranes of gram-negative bacteria (Nikaido and Vaara, 1985; Nikaido, 2003). Our previous studies have shown that both P44 and P28/Omp-1 have porin activities as measured by liposome swelling assay, allowing the diffusion of L-glutamine, monosaccharides arabinose and glucose, disaccharide sucrose, and even tetrasaccharide stachyose (Huang et al., 2007; Kumagai et al., 2008). Since the tricarboxylic acid (TCA) cycle in *A. phagocytophilum* and *E. chaffeensis* is incomplete (Dunning Hotopp et al., 2006), porin activity of P44 and P28/Omp-1 likely feeds the TCA cycle, and the differential expression of P44 or P28/OMP-1s might influence individual bacterial physiological activity (Huang et al., 2007; Kumagai et al., 2008).

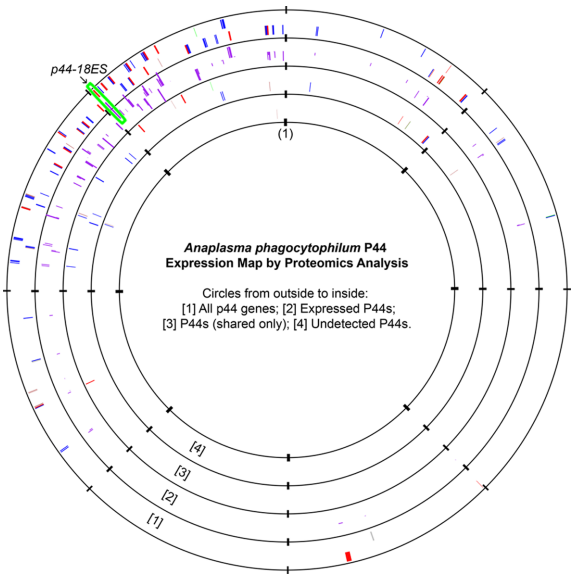

FIGURE 1 | *Anaplasma phagocytophilum* P44 expression maps as detected by proteomic analysis. All genes encoding P44 outer membrane proteins were plotted on the first circle. The bar heights on the second circle represented the number of P44-matching peptides detected, with higher bars indicating greater numbers of matching unique peptides. The third circle showed P44 proteins that had matched peptides but no unique peptide matches, and the fourth circle showed P44 proteins that had no peptide matches by proteomic analysis (APH_1122/P44-75, APH_1124/P44-C, and APH_1399/P44-C). The origin of the *A. phagocytophilum* genome was marked as (1), and the expression locus *p44-18ES* was highlighted by the green box. Color codes in circles 1, 2, and 4: Red, full-length *p44s*; Blue, truncated *p44s*; Green: N-terminal *p44* fragments; Brown: C-terminal *p44* fragments; Gray: Degenerated P44 fragments.

QUANTITATIVE ANALYSES OF PROTEIN EXPRESSION PROFILES IN *A. PHAGOCYTOPHILUM* AND *E. CHAFFEENSIS*

Following the determination of global expression profiling of these intracellular bacteria, we further determined the relative abundance of *A. phagocytophilum* and *E. chaffeensis* proteins expressed in human host cells. Quantitative analyses of protein expression were determined by averaging individual peptide abundances for the matching protein in the entire pool of peptides identified. Although different proteins do not contain the same peptides and protein abundances are not directly comparable, the relative correlation to the total abundance still exists, especially with at least threefold difference between proteins (Old et al., 2005). Quantitative analyses identified 130 proteins from *A. phagocytophilum* and 116 from *E. chaffeensis* as having relative abundances greater than 1 (**Table 4**; **Tables S6–S8** in Supplementary Material). Among them, the most abundant proteins detected are outer membrane proteins like *A. phagocytophilum* P44s and *E. chaffeensis* P28/Omp-1, RecF of *A. phagocytophilum*, chaperones like GroESL and DnaK involved in protein folding/stabilization, T4S apparatus, and enzymes involved in energy metabolism, transcription, protein synthesis, and biosynthesis of cofactors, nucleotides, and phospholipids (**Tables S6–S8**

Table 4 | Quantitation analysis of proteins in high abundance classified by functional categories[1].

Organisms	*Anaplasma phagocytophilum*	*Ehrlichia chaffeensis*
Transcription, amino acid biosynthesis, and protein synthesis	**20**	**42**
Biosynthesis of cofactors and nucleotides	10	10
DNA replication, recombination, and repair	5	2
Energy metabolism	**4**	**12**
Protein fate	7	11
Regulatory functions	1	2
Adaptation and detoxification	2	3
Transport and binding proteins	2	0
Cell envelope	**63**	**14**
Hypothetical proteins	16	20
Total numbers	130	116

[1]*Quantitative analysis identified 219 A. phagocytophilum and 255 E. chaffeensis proteins that have relative abundance values range from 0.17~14.5 and to 0.08~26.0, respectively (summarized in* ***Tables S6–S8*** *in Supplementary Material). Proteins with relative abundance value of greater than 1 were chosen for this analysis. The rows highlighted in bold indicate the role categories that show greater than two-fold differences in numbers of abundant proteins between two organisms.*

in Supplementary Material). Classification by functional role categories showed that *A. phagocytophilum* and *E. chaffeensis* have similar numbers of abundant proteins in all but three functional categories (**Table 4**, in bold font). Due to the expansion in P44 outer membrane family proteins, more proteins are expressed abundantly by *A. phagocytophilum* in the "Cell envelope" category. On the other hand, *E. chaffeensis* abundantly expresses more proteins involved in the categories including "Protein synthesis," like ribosomal proteins and "Energy metabolism," like electron transport chain proteins, probably because *E. chaffeensis* has additional ability to synthesize arginine and lysine but *A. phagocytophilum* does not (Dunning Hotopp et al., 2006). Interestingly, greater than 12% of these abundantly expressed proteins are hypothetical proteins or proteins with unknown functions (**Table 4**), suggesting that these proteins might be required for infecting human host cells and could be novel targets for the study of pathogenic mechanisms in human infection.

QUANTITATIVE ANALYSES OF UP- OR DOWN-REGULATED HUMAN PROTEINS IN *A. PHAGOCYTOPHILUM* AND *E. CHAFFEENSIS*-INFECTED HL-60 CELLS VS. UNINFECTED CELLS

As obligatory intracellular bacteria, the life cycles of *A. phagocytophilum* and *E. chaffeensis* are dependent on their mammalian hosts and are known to regulate or hijack host components for their survival (Rikihisa, 2010a,b). We, therefore determined the relative abundance of human proteins by comparing the LC–MS peptide peak intensity information of the same peptides from infected HL-60 cells to that from uninfected cells. A total of 48,054 human proteins were identified from HL-60 cells (**Table S9** in Supplementary Material). Quantitative analyses of human proteins in infected vs. uninfected HL-60 cells showed that infection by *A. phagocytophilum* and *E. chaffeensis* up-regulated the expression of proteins involved mostly in vesicular trafficking and cytoskeleton components, protein tyrosine kinases, pro-survival proteins, and enzymes involved in metabolism and oxidative respiration (**Table 5**; **Tables S10 and S11** in Supplementary Material). However, some proteins involved in host immune responses were down-regulated, including pattern recognition receptors like TLR1 and mannose receptor 2 (**Table 6**; **Tables S12 and S13** in Supplementary Material).

Several human genes that were up- or down-regulated by infection with *A. phagocytophilum* or *E. chaffeensis* have been reported previously. Up-regulated genes in human neutrophils at early stage of *A. phagocytophilum* infection included those that promote actin polymerization (Sukumaran et al., 2005). Up-regulation of genes involved in iron metabolism like transferrin-receptor was detected in *A. phagocytophilum*-infected NB4 cell, a human promyelocytic leukemia cell line (Pedra et al., 2005), and *E. chaffeensis*-infected THP-1 cell, a human monocytic leukemia cell line (Barnewall et al., 1999). The expression of histone deacetylase (HDAC) 1/2 was increased in *A. phagocytophilum*-infected THP-1 cells (Garcia-Garcia et al., 2009). Down-regulation of TLR2/4 mRNA and protein expression was reported in *E. chaffeensis*-infected human monocytes (Lin and Rikihisa, 2004). In addition, several reports have demonstrated the interactions between these up-regulated human proteins and bacterial proteins or activation of human proteins by bacterial infection. For example, the protein tyrosine kinase Fyn was shown to interact with *E. chaffeensis* TRP47 protein in THP-1 cells (Wakeel et al., 2009), whereas *A. phagocytophilum* induced actin phosphorylation by p21-activated kinase (PAK1) in *Ixodes* ticks (Sultana et al., 2010). *A. phagocytophilum*-containing morulae were colocalized with several

Table 5 | Up-regulated human proteins in infected vs. uninfected HL-60 cells by quantitative proteomics analysis[1].

Functional role category	*Anaplasma phagocytophilum*-infected vs. uninfected HL-60 cells	*Ehrlichia chaffeensis*-Infected vs. Uninfected HL-60 cells
Cytoskeleton components	Keratin; Arp2/3 protein complex; α-actinin-4; galectin-9; plastin-2	Actin; vimentin; α-actinin-4; Arp2/3 protein complex; galectin-9; keratin; ciliary rootlet coiled-coil protein; kinesin-like protein KIF17; plectin 1 (intermediate filament binding protein); plastin-2
Vesicular trafficking (ARF, Rab/Rho GTPases)	ADP-ribosylation factor (ARF) 1/3/4/5; ARF GTPase-activating protein GIT2; Rab 5/7/11/27; Rap1; Rho/Rac GEF 2; cell division cycle 42 (CDC42); transferrin-receptor protein 1; clathrin heavy chain; diaphanous homolog (mDia) 1	ADP-ribosylation factor (ARF) 1/3/4/5; ARF GTPase-activating protein GIT2; Rab 1/5/7/8/10/11/35; Rho-associated protein kinase 2; Rap1; Rho/Rac GEF 2; cell division cycle 42 (CDC42); STE20-like kinase; citron (Rho-interacting, ser/thr kinase 21); integrin-linked kinase; transferrin-receptor protein; clathrin heavy chain; mDia 1
Signal transduction (protein kinases and phosphatases)	Protein tyrosine kinase (Fyn/Lck); Ser/Thr-protein kinase PAK; P21-activated kinase (PAK) 2; casein kinase 2; sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA); fibroblast growth factor receptor (FGFR) 2; histone deacetylase 1/2	Protein tyrosine kinase (Fyn/Lck); Ser/Thr-protein kinase PAK; P21-activated kinase PAK2; casein kinase 2; sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA); Ca^{2+}/calmodulin-dependent protein kinase; fibroblast growth factor receptor (FGFR) 2
Immune response	MHC class I antigen; Fc fragment of IgE gamma	MHC class I antigen; Fc fragment of IgE gamma
Metabolism	Enolase; adenosine kinase; phosphofructokinase; pantothenate kinase (CoA Biosynthesis); cytochrome *b5* reductase; cytochrome *c* oxidase; NAD(P) dependent steroid dehydrogenase	Phosphofructokinase; adenosine kinase; pyridoxal (vitamin B6) kinase; pyruvate kinase; deoxycytidine kinase; NADH dehydrogenase; cytochrome *b5* reductase; cytochrome *c* oxidase; manganese-superoxide dismutase (SOD)
Cell-cycle regulation	Cyclin-dependent kinase 20	Cyclin-dependent kinase 20; apoptosis inhibitor 5

[1]Average abundance of human proteins was determined by comparing the LC–MS peptide peak intensity information of the same peptides from infected HL-60 cells to that from uninfected cells. Proteins with ratios greater than 2 (816 A. phagocytophilum and 1053 E. chaffeensis proteins) were identified and summarized in ***Tables S10 and S11*** *in Supplementary Material. Only proteins relevant to critical pathways affected by bacterial infection or with functions characterized previously were listed in this summary table.*

Table 6 | Down-regulated human proteins in infected vs. uninfected HL-60 cells by quantitative proteomics analysis[1].

Functional role category	*Anaplasma phagocytophilum*-infected vs. uninfected HL-60 cells	*Ehrlichia chaffeensis*-Infected vs. Uninfected HL-60 cells
Cytoskeleton	Kinesin-like protein 2; cofilin	Protein hook homolog
Immune response	Toll-like receptor (TLR) 1; macrophage mannose receptor 2	TLR-1; macrophage mannose receptor 2; oxidation resistance protein, complement control module
Signaling transduction	Protein tyrosine phosphatase isoform 3	Protein tyrosine phosphatase (non-receptor type 7)
Vesicular trafficking	Rap GEF	Rap GEF
Pro-apoptosis	Bcl-XL-binding protein	Pyrin-like protein

[1]Average abundance of human proteins was determined as described previously. Proteins with ratios less than 0.5 (343 A. phagocytophilum and 120 E. chaffeensis proteins) were identified and summarized in ***Tables S12 and S13*** *in Supplementary Material. Only proteins relevant to critical pathways affected by bacterial infection or with functions characterized previously were listed in this summary table.*

Rab GTPases, including Rab11 (Huang et al., 2010a), and *E. chaffeensis*-containing morulae were colocalized with Rab5 (Mott et al., 1999). Both *A. phagocytophilum*- and *E. chaffeensis*-containing morulae were colocalized with major histocompatibility complex (MHC) class I and II antigens (Mott et al., 1999). Several isoforms of sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) were up-regulated in *A. phagocytophilum*- and *E. chaffeensis*-infected HL-60 cells, suggesting proteins involved in the intracellular Ca^{2+} regulation like phospholipase C and transglutaminase shown in previous studies are critical in bacterial infection (Lin et al., 2002; de la Fuente et al., 2005).

There are several studies using microarray analyses to identify genes differentially regulated in response to *A. phagocytophilum* infection in human neutrophils and the promyelocytic leukemia cell lines NB4 and HL-60 cells at different infection stages (Borjesson et al., 2005; de la Fuente et al., 2005; Pedra et al., 2005; Sukumaran et al., 2005; Lee and Goodman, 2006; Galindo et al., 2008; Lee et al., 2008). These studies identified similar sets of differentially regulated genes involved in vesicular transport, cytoskeletal remodeling, signaling and communication events, cell-cycle and apoptosis regulation, and innate immunity. However, due to the differences in host cell types, efficiency of infection, post-infection time points, experimental designs, array platforms, databases used, and statistical analyses, a large portion of the genes are difficult to compare among these studies (Pedra et al., 2005; Lee et al., 2008). Since most cell functions are carried out by proteins, the comparison of proteomic data would reflect a more accurate state of cellular physiology and pathology. Nevertheless, combining these microarray and quantitative proteomic data would allow more comprehensive understanding of host cellular changes induced by infection with these pathogens. Our proteomic analyses reveal that infection with *A. phagocytophilum* or *E. chaffeensis* could modulate human host cell machinery to produce more energy, enhance vesicular transport, and activate cell signaling events involved in bacterial entry and proliferation. Further analyses of these up- and down-regulated human proteins will provide more information about the global regulation of host cells by infection with these intracellular pathogens.

CONCLUSION

The determination of bacterial proteomes is an important step in converting genetic information to protein function and cell biology. This study provides the first comprehensive proteomes of obligatory intracellular pathogens. A total of 1,212 *A. phagocytophilum* and 1,021 *E. chaffeensis* proteins are identified, representing 89.3 and 92.3% of the predicted bacterial proteomes, respectively. Nearly all proteins that have functions assigned are expressed in infected human hosts, including those involved in metabolism, pathogenesis, and regulation. Bacterial infection up-regulated the expression of human proteins involved mostly in cytoskeleton components, vesicular trafficking, cell signaling, and energy metabolism, but down-regulated some pattern recognition receptors involved in innate immunity. The availability of these proteomic data will provide a wealth of information on the molecular mechanisms of bacterial pathogenesis and therefore will greatly facilitate the understanding of the biology of these ehrlichiosis agents and the signaling events between intracellular bacteria and their host cells.

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SUPPLEMENTARY MATERIAL

Tables S1–S14 can be found online at http://www.frontiersin.org/Cellular_and_Infection_Microbiology/10.3389/fmicb.2011.00024/

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APPENDIX

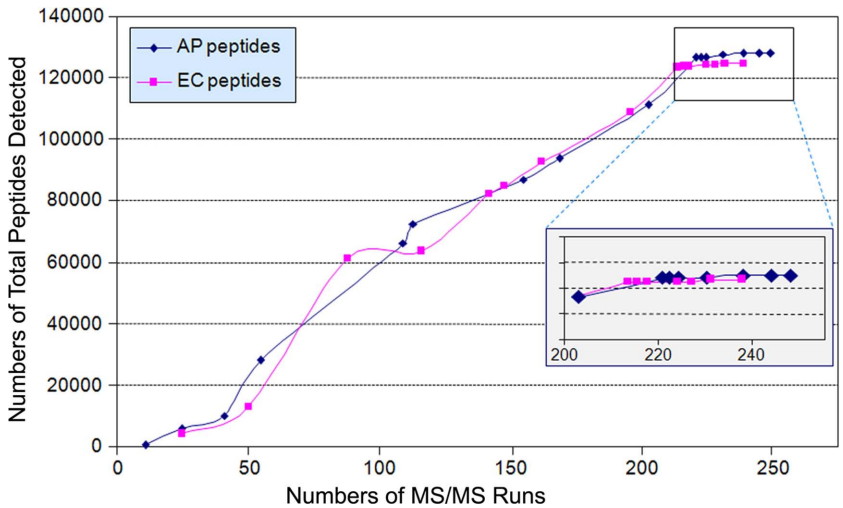

FIGURE A1 | Mass tag accumulation trends. Two AMT tag databases were constructed for positively-identified peptides, with numbers of total peptides accumulated versus total numbers of MS/MS runs were plotted. More than 126,000 peptides each were identified in protein samples from *A. phagocytophilum or E. chaffeensis,* using both purified bacteria from infected cells or whole lysates from infected cells.

A

Description	Frame	Overlap	Start	Stop
APH_0143 | P44-81 outer membrane protein, truncation	1	**204**	147820	147850
APH_0144 | P44 outer membrane protein, C-terminal fragment	1	**204**	147835	148038

Detection of peptides by proteomics: [1]

APH_0143 (76 aa):

EYKTTLKSEPN**TKFPTDISHGEISNSSILRATCRTSIIYKTLSRQIIINTISNNPMVEATR**KSR**DFRRELITQPSL**

APH_0144 (67 aa):

MS**YQLSPEISAFAGGFYHRVVGDGVYDDLPAQRLVDDTSPAGR**SK**DTAIANFSMAYVGGEFGVR**FAF

[1] Fonts in bold and red indicate peptide sequences detected by proteomics analysis.

FIGURE A2 | Continued

B

Description	Frame	Overlap	Start	Stop
APH_0063 | hypothetical protein	3	**49**	70473	70634
APH_0064 | transcription termination factor Rho	2	**49**	70586	71944
APH_0408 | glutamyl-tRNA synthetase	-2	**35**	425393	426799
APH_0409 | hypothetical protein	3	**35**	426765	426902
APH_0560 | ribonucleoside-diphosphate reductase, beta subunit	3	**50**	588435	589406
APH_0561 | putative thiol-disulfide oxidoreductase	1	**50**	589357	589989
APH_0640 | hypothetical protein	2	**50**	663791	663901
APH_0641 | hypothetical protein	-3	**50**	663852	664418
APH_0852 | putative glutamine-dependent NAD(+) synthetase	3	**40**	904563	906365
APH_0853 | hypothetical protein	-2	**40**	906326	906619
APH_1094 | hypothetical protein	2	**32**	1157366	1157494
APH_1095 | peptidase domain protein	3	**32**	1157463	1157687
APH_1140 | hypothetical protein	-1	**32**	1201810	1201992
APH_1141 | hypothetical protein	-2	**32**	1201961	1202059
APH_1171 | conserved hypothetical protein	1	**80**	1239985	1240662
APH_1172 | P44 outer membrane protein, C-terminal fragment	-2	**80**	1240583	1240819
APH_1235 | hypothetical protein	1	**70**	1305649	1306053
APH_1236 | hypothetical protein	3	**70**	1305984	1306127
APH_1237 | hypothetical protein	-3	**36**	1306092	1306226
APH_1322 | exodeoxyribo-nuclease VII, large subunit	-2	**32**	1389044	1390240
APH_1323 | zinc finger-like domain protein	-3	**32**	390209	1390826

FIGURE A2 | Continued

Detection of peptides by proteomics:

APH_0063 (53 aa):
MRGASLEVSILSQCRFVYMLGSIQDLASSDCEFLKVVLCVARLGEGKRLFLV

APH_0064 (452 aa):
MCGKVRRRKKAVPSAVVAEEATGSGSEEGRILNLCELKRKSTGELLAIAEELGVVSNGRMLKQEIIFQLMKRVISEGGVAIGGGV
VETLPDGFGFLRSAEANYAASSDDIYISAGQIKKFNLRTGDIVSGEIRAPGDKERYFTLVKAYSINYTEIGKLQRYVHFDDLIPV
YPEDRILLECNKGAGNEKKDISMRAIDIIAPLGKGQRALIVAPPRVGKTVILQQIAHSIAVNHPNMELIVLLIGERPEEVTDMLR
SVKGEVVSSTFDEPAYRHVQLAEIVIERAKRMVEHKKEVVILLDSITRLARAYNEVMPSSGKVLTGGVDSNALQRPKRFFGAARN
IENGGSLTIIATALIETGSKMDEVIFEEFKGTGNCEIILDRKIADKRIYPAIDISKSGTRKEDMLIESALLKKVWLLRRLLSAMG
PVEAMEFLRDKLSMAKDNNDFFEMMNS

APH_0408 (468 aa):
MRVVTRFAPSPTGSLHLGGARTALFNWLFARHHKGKFLLRMEDTDKKRSSDVVVQSIIDDMSWLGLQHDGDIVVQSSRAARHVAV
ARELVELGRAYRCYCSEDEVNEQKLQSEGTGKYFRHVCPWKHLNSTGDLPNKPYVVRLKSPENTTIEFLDGVYGKISVKSDQIDD
MVILRSDGTPTYLLAVVVDDHDMEITHIIRGSDHITNTVKQIVLAEAMSWVSPKFFHIPLIHDENGAKLSKRNRAPGIHEYKEQG
FLPEALCNYLLRMGWSYQNKEIVSMQEAIALFSMEDVGVSCSCLDYKKLVFLNHHYMGSKSESEILDLLLPILEEKLGGRISEEK
LSRLSLGIKQLVERAKTLTDLAEDSLFYVQDVEININPEAVETIQNSKKFLAELLESMSGIHPDMWKKTHLSSQIKEFSKTRNLA
MSDVYHFLRASITGRLQSPNISEVMEILGQEMCINRMLSAQEI

APH_0409 (45 aa):
MGDGANLVTTRTAVDLLKIHRGVMVQRKKTNSNALAGELKYYTET

APH_0560 (323 aa):
MSLLDAKPVYKPFDYPWAYDAWLCQQRIHWLPEEVPLADDVQDWKTMLSEKEKNLLVQIFRFFTQADIEVNNCYMKHYSNIFRPT
EICMMLSAFSNMETIHVAAYSYLLDTIGMPEVEYQAFLKFEVMKKKYDYMQRFEECRRGDKRHVAKTLAVFGAFTEGLQLFASFA
ILLNFQRFGKMKGMGQIIAWSARDESLHTDSIIRLFNTFVHENGEIWDDDLKQELYDACKVIVMLEDEFIDLAFALGDVEGLSAC
EVKRYIRFIANRRLKQLHLEPIYDVAENPLPWLDEILNGVEHTNFFENRVTEYARAATEGTWEEAFED

APH_0561 (210 aa):
MLELQRREPGRKRLRISKWLTCAVAALLVLSCYAHAAEQVLQHRRVQEILPRVFVEGKVSRGFKVSNKFLADLDGTELTLRDIAQ
DRVCVIVFWAPWNLDSVMLLQGIQRVSENLAAKDLGDTVVFLPISDVGIDDVPKVLRARDSYGLTLPMYIDVKHELFDYFDVTAI
PLTLIVNRKGEVIYRIVGYMQWENTAVENELLSIVNQAQE

APH_0640 (36 aa):
MQIGLERQESDKWIDHLVFSAIVKNQIFFAKAPKEE

APH_0641 (188 aa):
MQRKRVQKQLPGYLEQHKSFKKKRTPLSSTALSYTEREKIKKRERNAQQYRCNIAFQKPTNTSFVLRLTEQQRGVSAVIQRMRKV
TIAHGMDPVQRAGQTKSSNEKDAARFGRVPKGKKRTSAMSFLKMLLKFLLMLLFPIMFCSNLIVKGILKDLQSRHKPTKKTSRRP
FSAILLLGPSRRKFGFSR

APH_0852 (600 aa):
MTRILVKQLNYGPNAVHSNCERILAECREASAGDIVLFSRYAVCGYFDKAPLLSGNLLQLCNKHLESLASSIGSACAIIGGLALQ
DGVLTEAIYLISDGNYEKLIELPISSYNAAEKHIVLSLGGLRIALLLEEHTAHSNAQTWHGAYQNVPTDIDVLILLGRSQYSYYS
LLSSNAAISIMQNHRAFIYLNILGGYGSDVFAGGSLIHDGINATTLALCKEDARIIEITATNSIVTCADGVPRAHSEHISDHNCT
SGISASENLTLDASPTSERSVTEGVLSTSKHVEQSPNIGVLESHTLNAHSQLHSLKTDKVFSLAELKSLNMDWDVFTYQMLVLAL
RDYVKKSGFSGVLLGLSGGIDSALVAAIASDALGAEHVHTFMLTTRHTSQSSVTDAQRCAELLGTHHEVVSIEEAFCTCIESLKT
YIDTPTPNNALENMQSRIRGMYLMAISNANSLLLLATGNKSELLTGYMTLYGDTCGGYAPINNVYKTKVYDLVKWRNSNIPANSL

FIGURE A2 | Continued

CRKMHVIPENIITKAPSAELKPNQTDQDTLPEYDKLDGILSLLVDRFATREDIVLSGYTEEEVDLVMNLVKKSAFKLDQVAPGPD
IRAKS

APH_0853 (97 aa):
MLIDDHAADFFCKIICTFKVAKRFSSVAEYVLLQINTNLYPGARSSRKGMALSEINRMSTQFYAVQPTSRDCGIKKCQHSSSFVF
YDLARMSGPGAT

APH_1094 (42 aa):
MFAKSFLSLFPRVLCVQKLFPMQRKSISQRLIIENYHASGRG

APH_1095 (75 aa):
MRIIMLQGADENQKLYFLVLSDGHCQLMLAHDIGNYSKLGDAIDDSLDEAFDKECLGQGIRVVWIPMLRYFELRQ

APH_1140 (60 aa):
MDCPALSSEARVTSGNYWLTALTPISGAITASAAIDLEDHNLYTFRSAITRNKRSAFMLA

APH_1141 (32 aa):
MSYGALMQMAIVALSKKAAALPHGLPSTVIRS

APH_1171 (225 aa):
MRFGSIIFKIFGCVFPCAILLTLGTWQILRLQEKLHIIHTMSGAIVPLPEGDDLQSHNYKRIQVQGTFKTTYFRVFAGRAGYYFL
QPMELTDGRHVLINRGTLSEYAKIDIQDASMDEQVSGTLYCTLSSKTKWVAANNADKNLWFWYDIESMSKHIGVPLEDCIIWGDK
TSLLDGLQPNKMPQVRNDHLEYAITWYTLAMIWVGGYIYFLRTRQRLRSRPHNPE

APH_1172 (78 aa):
ICALHIDLCIPRASVINLIIKAKTGVSVRRLYIVPYACVGLGGNFVGVVDGHITLGYEVYCATAALFSENKYNPLPIS

APH_1235 (134 aa):
MKGKSDSEIRTSSSIRTSSSDDSRSSDDSTRIRASKTHPQAPSDNSSILSSEDIESVMRCLEEEYGQKLSSELKKSMREEISTAV
PELTRALIPLLASASDSDSSSRKLQEEWVKTFMAIMLPHMQKIVASTQG

APH_1236 (47 aa):
MGENIHGYYVAAYAENCGIDPRLGLAQETAAVQAQRLTGSRCCMHSQ

APH_1237 (44 aa):
MWKKESEENCELGMGEMPGAMLLDLLQASIKQHLLTVHAAPAAG

APH_1322 (398 aa):
MQVTLLRSFSNDIPEFTVTEITGVLQRFMQETFYSIKVRGEISGLSRPNSGHVYFTLKDSNSVINAVCWNGTRLKVQFCDGLEVV
CTGYLSVYQSKYQLIVTDMTLAGYGKLAAMLAELKKKLELEGLFSPARKKKLPFLPTKIGVITSPTGAVISDIISRVKQRFPSNV
VVWPVQVQGDRASAMVIEAIKGFNSFADPPHVIIVARGGGSFEDLWPFNDEELARTVAASKIPIVSAIGHETDFTIIDYAADLRA
STPTAAVELVLPEKSKLVASINEKFVRTKISFERIVKMQEYRLLRLHGILTEKKNSLLQKSRVALEYQQKIRYLLQVSLLRKRQY
LESLMQRLSYYDSKHILSVGYAIVRDEHEKQISSVEALSTNDTITIELKDGKRRAIII

APH_1323 (205aa):
MIRVVCTNCSAVYSVAGTRIPKKGKEVKCSHCHHTWLFMPENVSIPSKGPPGGKKERVEKFFWGKTLIQMIILFPLLFFFSSSFQ
DRFSYTFRKIYRLTEIYDTSDIKLRSSGVEVLEVHGDGTMQVRVRWIIINNADKERFVPDVRFTFYDENQKSVFSKKIEVDKYNV
IKSKTGMHFERVIEGVPSSANTVQVRAGNAFEIFF

FIGURE A2 | Expression of overlapping ORFs in *A. phagocytophilum*. **(A)** Expression of complete overlapping protein-coding ORFs in *A. phagocytophilum*. **(B)** Expression of partial overlapping protein-coding ORFs in *A. phagocytophilum*. Peptide sequences detected by proteomics analysis were highlighted by bold and red fonts in amino acid sequences for these overlapping ORFs.

A

Description	Frame	Overlap	Start	Stop
ECH_0506 | hypothetical protein	-3	**120**	509421	509711
ECH_0507 | hypothetical protein	2	**120**	509561	509680

Detection of peptides by proteomics: [1]

ECH_0506 (96 aa):

MLQFKILSCKAIVLLLS**MYSILCFQYLNFYR**ISQHNKIYTSLLNNVFYSSKLLFEYFYTILNTLFITSKH
TIITKSTISHYKSHINLNITDFQRLN

ECH_0507 (39 aa):

MMS**KIHYLK**VKYRFCYAGIFYKNLST**ESRVLNTLIGVEQ**

B

Description	Frame	Overlap	Start	Stop
ECH_0472 | hypothetical protein	3	**81**	450456	450590
ECH_1158 | 6SRNA1	3	**81**	450510	450671

Detection of peptides by proteomics:

ECH_0472 (44 aa):

MSYVTIELIGPLYCVRIA**LGVSVLYVEVKLIPRARLIFLGSCHC**

[1] Fonts in bold and red indicate peptide sequences detected by proteomics analysis.

FIGURE A3 | Continued

C

Description	Frame	Overlap	Start	Stop
ECH0086 | hypothetical protein	-2	**119**	76895 ←	77050
ECH0087 | hypothetical protein	3	**119**	76932 →	78782
ECH0113 | hypothetical protein	2	**243**	98684 →	101065
ECH0114 | hypothetical protein	-2	**243**	100823 ←	101191
ECH0253 | hypothetical protein	-3	**107**	239628 ←	240197
ECH0254 | hypothetical protein	1	**107**	240091 →	240219
ECH0344 | hypothetical protein	2	**115**	334856 →	335086
ECH0345 | conserved hypothetical protein	-1	**115**	334972 ←	335856

Detection of peptides by proteomics:

ECH_0086 (51 aa):

MSSASKDSNMLINDGSKFNILLNIRGLVYLAIAILRI**INILIPNYIK**LLHI

ECH_0087 (616 aa):

MLIMR**RMAIARYTKPLI**FSKILNFDPSFINMLESLEAEDILLLKDELGSVFYYLIMSLYDVSGI**QNDSSG**
AIRERLKRVLLDAIEQS**NSDQSGAVGSQVSEIREIRDRLR**SAYQSISNR**VCRTLISALGNETLQEETDSN**
ITQQSNLQRRLQM**FQYVIQAVEILADKLNTAVVEGKVSPEQVSEYLSCTNESHDSIAPDTMSALVKLYSL**
TDDPQLTDLAYSLVKTFLMKFGRYKLDGQGRNSMHYAVNMCSPERQESFLCEMIQPSIYERVSIVNEVDA
SRNNLMHYAACAPYMNYQILKYLVKNFPAMMTQQNCYGDTPLHIMSYVYFVNFAKILSSY**NITYRENMNA**
LKEVVDRGLPLSQMR**ERVMSIRRNDEALSRQLKA**YVDESV**GTYQLLLTMVPLR**QIFEVRNNAGHTVYDIM
NASMSNIGNER**LEALLQDFSQASSRLPIYDCRI**DSQHELCVNLCFSNKYRVVGKSGYGHVLYTHVKRMYD
LISYKFSEIS**DSRMRCIKIENERGNNRYLSMLVVMMLMALCVLNTVLHFK**TRSILGIEQGLYRSVLFSAI
SVVFFVSICVFCIVYAKYVDVADK**KLIIEEEGYARSILLSHLDVQETDTSQRR**REG

ECH_0113 (793 aa):

MRTGIVVGVNEQDIAGEEQEMPLSQQFPNLHPESCQDIIKAEDRRVGVVTKKKKRK**SSPVSKDMIRSSDQ**
LSTGTLKESDEYGKKRKSKNR**KTGSPLKQVKTVIVVGTNKQDIASEEQ**EMPLSQQLS**DLHPESCDQDIVK**
AEDRRVCVATKEKRRKSSPISK**DMIGSSDQLSIGTLKES**DEYGKK**RKSKNRKTGSPLKQVKTGIVASANE**
QDIAGEGQGIPLSHQLLHFESCDQNIVKVEDRKVHVVTREERK**SNPVLEDNEYLKQDAK**SKVHAGQAVGQ
PMSCAEKAKAK**VSSVEETVGKVCTDQKVKE**VSECKNDVYDTSVQHTAVSYVEKVKVGLSDDL**GKTLLPID**
LKRTVSQQDLNSEYLKQSAKSKVHAGQAVGQPMSYAEK**AKVSSVKEAVGKACTGQKVKGA**SGFKNDVDET
LSASMQHTAVPGMVTPTNIIPARHIVSGVAVFKHLQGYSLEEYQSLVENFRKSVACFSK**MHMSYAYQILH**
NTFIVHNGQIVLKPE**IEQFLLKTTSNIKLCTFIIKIGIV**DQVMARLGNSYGCNSIKYCASYYHDS**EIIDE**
ILIRFYGTSYSSFSEVSQQVRHDTVDCLKKRNVTNVFKLHDSSFYSEVLSVCCNFMGVSNKDIELHKLCS
FVQLSCCIQIAQMYHMMLKVKYS**AGDKQDYAQQCISER**LAKGPAIKDFMYRALLFGRCACNVRNTFRHLY

FIGURE A3 | Continued

SPDDKNHIRTVSGLNVPYC**MIRVSSKGIIPK**IESYIKRNKMDF**GVFVDDIVNFVR**DALYRPGERTCIKED
IKTYHTIVSSRYDNMISNCSVSR

ECH_0114 (122 aa):

MSFVFSSTQTPSPGDTTITYHVVYRLLIIEYMFFVLFYTNFFSSGNATITYHIVISTTYYSVICFYVFFD
TSSFTWTIQCIS**DKIYYIINK**NTKIHFISLNITLNFWNNSLTTYSNHTIRNV

ECH_0253 (189 aa):

MTNETNLTTNPTGALTTTQGTLSNITTPSSDTAEDMATTIF**YSIILIVVFMIAVVKLFFPTRTDPYNNLL
LDSETETET**GYSDSDTDLDSEENIPLLENAHLTLIDDEEEENIISLINLTEVHVTNTHGRNTDTNDTESN
NGLDDDDEIPLLLHGSLRLMNEEDNNVNPSVILTDVCTTNTQESGSIHK

ECH_0254 (42 aa):

MSSAVSLEGVVIFDN**VPCVVVSAPVGFVVK**FVSFVIIFYLIE

ECH_0344 (76 aa):

MCSIYVSYGNFGVQFIFVKYR**KTIKVRYQLLNEKFSKCIYE**MLVLLIVPPPASCCAINSTLLIFVKSCTE
SISLYI

ECH_0345 (294 aa):

M**NHFVVARRSDDSVISVQQNNTLQ**NLTISNLNKPAEILLEYTVEELSN**KPLSTILHKNIVENINSYLEYT
SDGTDLFDILSKTR**NCSFIGKN**NKAIPVTPK**VFRV**IASNQDIINYEILIR**DISISQKLDIFKESVIFNTK
YNMHPTFNIMDEASTKTEVQIILDFLHKYNTHAVISMIQLDPPHNSSNIDSLTQQTINLLH**KNIRESDII
GYIGNHK**IICILLGCK**SEHAYSAISR**IHK**NINNNLQDSHAKISVG**YAQMYNEIDSVQLLTNIS**NVLFIAQ
QEAGGGTIK**STNIS

ECH_0344 (76 aa):

MCSIYVSYGNFGVQFIFVKYR**KTIKVRYQLLNEKFSKCIYE**MLVLLIVPPPASCCAINSTLLIFVKSCTE
SISLYI

ECH_0345 (294 aa):

M**NHFVVARRSDDSVISVQQNNTLQ**NLTISNLNKPAEILLEYTVEELSN**KPLSTILHKNIVENINSYLEYT
SDGTDLFDILSKTR**NCSFIGKN**NKAIPVTPK**VFRV**IASNQDIINYEILIR**DISISQKLDIFKESVIFNTK
YNMHPTFNIMDEASTKTEVQIILDFLHKYNTHAVISMIQLDPPHNSSNIDSLTQQTINLLH**KNIRESDII
GYIGNHK**IICILLGCK**SEHAYSAISR**IHK**NINNNLQDSHAKISVG**YAQMYNEIDSVQLLTNIS**NVLFIAQ
QEAGGGTIK**STNIS

FIGURE A3 | Expression of overlapping ORFs in *E. chaffeensis*. **(A)** Expression of complete overlapping protein-coding ORFs in *E. chaffeensis*. **(B)** Expression of overlapping RNA and protein-coding ORFs in *E. chaffeensis*. **(C)** Expression of partial overlapping proteincoding ORFs in *E. chaffeensis*. Peptide sequences detected by proteomics analysis were highlighted by bold and red fonts in amino acid sequences for these overlapping ORFs.